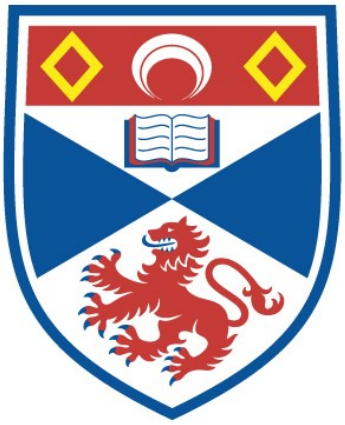
SOME STUDIES OF CLINICALLY SIGNIFICANT BINDING

Alexis Shona Roberts-McIntosh

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Some Studies of Clinically Significant Binding

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by

Alexis Shona Roberts-McIntosh

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United College of

St Salvators and St Leonards,

St Andrews.

Declaration for the Degree of Ph. D.

I, Alexis Shona Roberts-McIntosh, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfillment of any other degree or professional qualification.

Alexis Roberts-McIntosh 29th October 1986

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No 12 on 1st October 1983 and as a candidate for the degree of Ph. D. on 1st October 1984.

29th October 1986

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph. D.

Supervisor 29th October 1986

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Abstract

The work described in this thesis falls into two areas of clinically significant binding :- the interaction of sodium nitroprusside with biological macromolecules, and the consequence of bilirubin to albumin binding in the assay for bilirubin.

In Part 1; Chapter 1 is a general introduction reviewing the chemistry of sodium nitroprusside and its medical application. Chapter 2 is concerned with the evaluation of aquocobalamin as a cyanide trapping agent and the stability of sodium nitroprusside. Chapter 3 describes ^{13}C NMR and EHMO studies of the binding of cyanoferrates to cobalamins and cobaloximes. Chapter 4 reports on the pharmacological consequences of the interaction of nitroprusside with aquocobalamin. The second section of this chapter considers the interaction of nitroprusside with albumin and haemoglobins.

In Part 2 of this thesis Chapter 5 reviews the methods used in bilirubin assay. Chapter 6 describes fluorescence and solid state NMR studies investigating the binding of bilirubin to albumin and evaluates the ability of various "accelerators" to interfere with the binding of bilirubin to albumin in the diazo method of bilirubin assay. Chapter 7 considers the problems involved in using diazonium salts in the wet chemistry assays of bilirubin and describes an initial study evaluating the use of heterocyclic diazo compounds in the diazo dry base multi-layer film method of bilirubin assay.

To my Husband, Charles.

and my Mother, Mary

Part 1

Chapter 1

REVIEW OF SODIUM NITROPRUSSIDE CHEMISTRY AND MEDICAL APPLICATION

Abbreviations

SNP = Sodium Nitroprusside

Cyclic GMP = Guanosine-3',5'-monophosphate

1.1 INTRODUCTION

Sodium nitroprusside (SNP) has the formula $Na_2Fe(II)(CN)_5NO.2H_2O$. It exists as red-brown crystals and was first prepared by Playfair in 1850[1]: its chemistry has been reviewed by Swinehart.[2] The ion is generally regarded as containing Fe(II) and NO^+ species[3,4], but molecular orbital calculations on nitroprusside[5] give a charge distribution of $Fe^{+0.32}-NO^{+0.46}$.

In 1929 Johnson[6] first described the hypotensive action of the nitroprusside ion. It became established in the mid 1960's[7] as a potent rapidly acting vasodilator used in anaesthesia. In more recent years it has been used in cardiac surgery[8-10] and following myocardial infarction[11-13], as it causes relaxation of the arteries and thus reduces stress on the weakened heart. Controlled intravenous infusion of a solution of sodium nitroprusside can cause a fall in peripheral resistance and an increase in arterial capacity to any desired level within 30 seconds. The duration of its action is extremely short; on discontinuation of infusion the blood pressure rapidly returns to normal, usually within 2-5 minutes. It is thus easier to control the blood pressure than with any other hypotensive drug.[14,15] The nitroprusside anion is extremely water soluble and lipid insoluble and hence remains in the blood stream during action whereas organic hypotensive agents become associated to the lipid membranes causing problems with stabilisation ("overshoot") of blood pressure before and after the operation. Lack of "overshoot" is the major advantage nitroprusside has over its contemporary organic hypotensive agents.

1.2 MECHANISM OF SNP ACTION

SNP acts predominantly on the arterial pressure rather than the venous.[16] The physiological activity of nitroprusside is thought to be due to the reaction of the NO^+ with the thiol[17] groups in the cell receptor sites in the smooth muscle membrane and intracellular space. *In vitro* the NO^+ ligand initially reacts with thiols by nucleophilic attack at the nitrogen of the nitrosyl group.[18] *In vivo* the hypotensive action is thought to be due to the nitroprusside ion undergoing a *trans*-nitrosation reaction with the enzyme guanylate cyclase [GTP pyrophosphate lyase (cyclising) EC 4.6.1.2].[19] The mechanism is still a very controversial subject[20-22] but it has been established that guanosine-3`,5`-monophosphate[23] (cyclic GMP) is involved in the vascular smooth muscle relaxant response to nitrogen-oxide containing drugs.[24] The initial enzyme activation is thought by some workers[25] to require the presence of activated thiol groups. The vasodilator action is attributed to the formation of an active unstable intermediate, S-nitrosothiol[26] (S-nitrosocysteine), and it is the NO^+ which is the potent vascular smooth muscle relaxant.

Craven[27] first reported guanylate cyclase was insensitive to activation by NO^+ or nitroso compounds; the enzyme activation was restored in the presence of haemoproteins.[28-31] Recent observations[22] show that partially purified hepatic guanylate cyclase readily binds haem and NO-haem which suggests that haem may also be associated with, and related to, the mechanism of guanylate cyclase

activation by NO^+. Ignarro's studies[22] indicate the likely reason for the haem requirement is that NO and nitroso compounds such as SNP react with the haem to form NO-haem complexes[32] which bind to,[30] and activate[27] guanylate cyclase. Chapter 4 of this work studies in more depth the *in vitro* interaction of nitroprusside with different forms of haemoglobin.

1.3 PROBLEMS ASSOCIATED WITH MEDICAL APPLICATION

In spite of its value, there is a problem in the use of SNP. In recent years it has been reported that the nitroprusside ion has toxic side effects, releasing cyanide in the presence of whole blood both *in vivo*[32-35] and *in vitro*.[36-38] These reports claim that free cyanide has been detected in the blood plasma and in the exhaled air of patients. In addition thiocyanate, the product of cyanide metabolism was reported[34] to be present in abnormally high concentrations. Reports of fatalities after SNP infusion have come from Jack[39], Merrifield[40], and Davies[41] and each have three common factors:-

(i) metabolic acidosis was always present,

(ii) since all patients were resistant to SNP dosage as high as 750mg was infused,

(iii) death occurred in the immediate post anaesthetic period.

These reports resulted in conservative guidelines for maximum dosage being set down for users[42] - an upper limit of 50mg for an average 3 hour operation.

In view of the large formation constant for cyanoferrates,[43] the very high thermodynamic stability, and the known chemistry of sodium nitroprusside[2] these results are very surprising.

It is known[44] that a solution of SNP reacts photochemically to yield a blue precipitate of Prussian Blue after a few days.

$$[Fe(II)(CN)_5NO]^{2-} \xrightarrow{(i)} [Fe(III)(CN)_5H_2O]^{2-}$$

d^6 $\qquad$ d^5

$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ (ii) $\downarrow$

(i) H_2O, hv (400nm)

(ii) H^+ $\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ $Fe^{3+} + 5CN^- + H_2O$

Nitroprusside ions contain low spin Fe(II), a d^6 species, in which the ligands are kinetically inert. However, photolysis results in the formation of aquopentacyanoferrate(III). The electronic configuration of the iron is now d^5 and the ligands are now labile.

Previous extensive studies[45,46] using cyanide sensitive electrodes and excluding light totally showed that no cyanide was being released. This contrasts with those reports[38,47,48a,49] of SNP decomposition in the system.

The technique[47] used to isolate the cyanide for assay involves acidification of the cyanide containing solution, and removal of the volatile HCN thus formed by passage of a stream of nitrogen through the solution. The HCN is then trapped in a sodium hydroxide solution. The

disadvantage of this technique is its invasive nature. This conventional technique is unreliable for measuring the concentration of cyanide if the nitroprusside ion is also present, because of the time scale of determination. In the presence of light SNP will be photolysed[44] on a significant scale to form $[Fe(III)(CN)_5H_2O]^{2-}$, where the cyanide ligands, although labile, remain in equilibrium with the iron. It is the intrusive action of acidifying the solution and the procedure of passing a stream of nitrogen through the solution to remove any volatile HCN so formed that causes decomposition. Although $[Fe(III)(CN)_5H_2O]^{2-}$ is also a stable species (est. $\beta_5 = 10^{36}$),[43] as a result of this non-equilibrium process it will eventually release all of its cyanide as the labile ligands are protonated and swept out of solution, thus giving an apparent high cyanide concentration. Therefore, this conventional technique is excellent for solutions containing cyanide only, but is inadequate and misleading for solutions containing nitroprusside. Use of the cyanide electrode technique for measurement of the cyanide levels in alkaline solutions only measures concentrations down to 10^{-5} mol dm^{-3}. This is too high in comparison with concentrations of cyanide due to SNP decomposition that might be detected in blood during a three hour operation.

A second method of investigation has recently been employed in this department; a study of the ^{13}C NMR spectrum[50] of ^{13}C-labelled SNP in whole blood. This technique is rapid and non-invasive. SNP (90% ^{13}C) was added to the whole blood and the spectrum of the nitroprusside ion was recorded every hour for fourteen hours. The results showed that after fourteen hours no SNP decomposition was detected. Due to the accumulative scanning effect over this long period even a 5%

decomposition would have been detected. The study was carried out at SNP concentrations of 1.4mg ml^{-1} of whole blood, which is still rather high in comparison to concentrations found under operating conditions, but not very different from other *in vitro* studies. In an attempt to remove the limitation of sensitivity and develop an efficient method of detecting any breakdown of SNP the study described in Chapter 2 was undertaken.

As a result of the observations in Chapter 2, high field NMR and extended Huckel calculations were carried out and are reported in Chapter 3, and in Chapter 4 *in vivo* studies are reported which investigate the implication of these aforementioned observations.

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Chapter 2

RADIATION AND UV-VISIBLE STUDIES OF THE *IN VITRO* STABILITY OF NITROPRUSSIDE

Abbreviations

SNP = Sodium Nitroprusside

H_2OCb = Aquocobalamin

CNCb = Cyanocobalamin

2.1 INTRODUCTION

In the 1960's sodium nitroprusside was first introduced into the UK as a hypotensive agent during surgery.[1] It causes lowering of the blood pressure by dilation of the blood vessels. In the last twenty years this vasodilator has been extensively used since it possesses one major advantage over other hypotensive agents; when administration of the dose is terminated after surgery the blood pressure returns rapidly to normal.[2] With other hypotensive drugs the blood pressure "overshoots" the normal level.

Over the years there have been several reports of cyanide being released when SNP is administered during surgery.[3-7] Although the extent to which this cyanide is released in the blood has been disputed[8,9], it is presumed to be released in the body and to account for the acute toxicity of SNP.[10,11] As a consequence a low maximum dosage has been recommended.[12,13]

The nitroprusside ion is a very stable species - from the known formation constant[14] of $[Fe(II)(CN)_6]^{4-}$ and $[Fe(III)(CN)_6]^{3-}$ it can be estimated that nitroprusside has $\beta_5 = 10^{36}$. Therefore, from a thermodynamic viewpoint, a reaction involving the complete breakdown of nitroprusside with liberation of cyanide is very surprising. Furthermore, reactions of nitroprusside generally involve the nitrosyl group, with the $Fe(CN)_5$ moiety remaining intact.

As discussed in Chapter 1, previous methods of detection of free cyanide using colourimetric techniques had major drawbacks.[3,15,16] The non-intrusive technique of ^{13}C NMR[17] showed no reaction of SNP with whole blood, but the detection limits for cyanide are too high. If free cyanide was produced at a level of only 10^{-4}-10^{-5} mol dm^{-1}, it would not have been detected using NMR methods.

To remove the limitation of sensitivity, and develop an efficient method of detecting any breakdown of SNP, experiments using ^{14}C-labelling were carried out. These were coupled with the development of an efficient method of extracting Vitamin B_{12} from whole blood.

If SNP decomposes *in vivo*[7] the liver is the site of detoxification where cyanide is converted to thiocyanate (see Figure 1). However, to prevent levels of cyanide greater than the liver can handle it is recommended that aquocobalamin (Vitamin B_{12a}) be used as an antidote.[18,19]

$$Na_2S_2O_3 + NaCN \xrightarrow{\text{Rhodanase}} NaSCN + Na_2SO_3$$

Figure 1 : Detoxification of Cyanide in the Liver.

Aquocobalamin is often referred to as hydroxocobalamin; but as it has a pK_a = 8.1 it is primarily in the aquo form[20] at physiological pH. At this stage it must be noted that while some reports indicate that the administration of H_2OCb alongside nitroprusside reduces levels of free cyanide in both red cells and plasma,[18,21,22] others

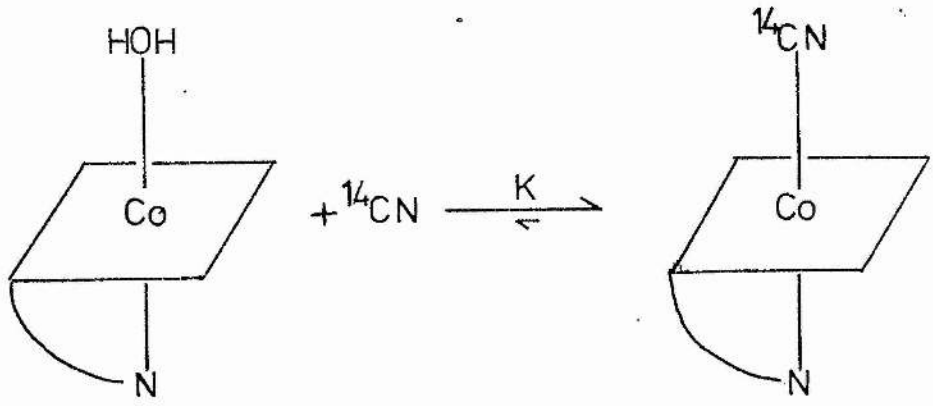
suggest that H_2OCb is a poor antidote.[23] It has also been reported that H_2OCb raises free cyanide levels during infusion of SNP.[24]

Mushett[25] first claimed that aquocobalamin (Vitamin B_{12a}) was capable of both preventing and reversing the toxic effects of cyanide *in vivo* if injected 20 seconds before cyanide infusion, or within 4 minutes thereafter. This produced cyanocobalamin (Vitamin B_{12}); a non-toxic substance which can readily be excreted. Rose[26] showed that intravenous injection of aquocobalamin tends to delay the onset of toxic signs, prolonging the survival time after the administration of lethal doses of cyanide. In more recent years[27,28] there have been reports recommending the future use of aquocobalamin as an antidote to lethal and chronic cyanide poisoning.[29]

From a chemical point of view the apparent antidotal action of aquocobalamin on lethal doses of cyanide is surprising. The reaction of cyanide (or hydrogen cyanide) with H_2OCb at physiological pH is undoubtedly irreversible, but fairly slow. For the formation of cyanocobalamin from H_2OCb Williams[30] reported a formation constant of $K > 10^{12}$, but found, at concentrations of 2.5×10^{-5} mol dm^{-1} at pH 4.5, a half-life for CNCb formation of some four and a half hours. A subsequent determination[31] gave $K = 1.2 \times 10^{14}$. The kinetics of the reactions of cyanide with H_2OCb were studied in great detail by Jencks[20]; although H_2OCb reacts with cyanide to form both mono- and dicyano derivatives, the conjugate base (hydroxocobalamin) does not react at all.[26]

The observed rate differences of the conversion of aquocobalamin to cyanocobalamin at physiological pH may be due to the differences in both whole blood and isotonic buffer, pH 7.2.

First, UV/visible spectroscopic studies in isotonic buffer, pH 7.2, were carried out to establish how rapid the reaction of cyanide and aquocobalamin is. Radioactive control experiments were then carried out to establish whether H_2OCb is in fact a good trapping agent for cyanide when incubated for one hour and to determine any differences in buffer and blood.



K (pH 7.2) = 10^{12} per cyano group.

Figure 2 : Essential Reaction Scheme

Isotopic dilution methods were then developed to extract any irreversibly formed radioactive cyanocobalamin from buffer and blood media.

The synthesis of $Na_2[Fe(^{14}CN)_5NO]$ was then undertaken and it's behaviour was studied in the presence of aquocobalamin, using the same incubation time, in both buffer and whole blood.

2.2 EXPERIMENTAL

2.2.1 PREPARATION OF SODIUM NITROPRUSSIDE-^{14}C

Materials and Method

Conversion of Sodium Hexacyanoferrate(II)-^{14}C to Sodium Nitroprusside-^{14}C

Sodium hexacyanoferrate(II)-^{14}C [$Na_4Fe(^{14}CN)_6.10H_2O$; 2.94mCi $mmol^{-1}$] was purchased from Amersham International. Sodium hexacyanoferrate(II) was obtained from BDH Chemicals Ltd and was of AnalaR grade.

Pye Unicam SP8-100 and Perkin Elmer 1310 spectrometers were employed for UV/visible and IR analysis respectively.

Initally the synthesis was carried out using non-radioactive ferrocyanide several times to maximise yields and to find the optimum small scale reaction conditions.

This synthesis is adapted from the industrial process which was kindly supplied by Mr. C. Garnsworthy of BDH Chemicals Ltd. This in itself is a modification of the method of Steel.[33]

Sodium hexacyanoferrate(II)-^{14}C [$Na_4Fe(II)(^{14}CN)_6.10H_2O$]; (2.5g, 50-70 μCi mmol^{-1}) was added to an ice-cold mixture of 2.25ml nitric acid (S.G. 1.42) and water (1ml) with stirring. More hexacyanoferrate(II)-^{14}C (1.54g), of the same specific activity, was added in portions over 4 hours and then the mixture was allowed to warm to room temperature over 1 hour. It was then heated to 60°C and sodium carbonate (0.22g) was added in portions over 15-30 minutes allowing the effervescence to die down somewhat between each addition. The mixture was then heated to 75°C and a further 0.18g sodium carbonate was added and stirred for 1 hour allowing it to cool to 65°C. After addition of a mixture of methanol (10ml) and water (3ml) the solution was allowed to stand at 50-60°C for 48 hours in the dark under a gentle stream of nitrogen. After 48 hours the mixture was filtered through a bed of Hyflo-Supercel (BDH Chemicals Ltd.), the reaction flask and Hyflo bed were washed with methanol until the washings were almost colourless. The resulting dark red filtrate was filtered again through a glass sinter and concentrated on a rotary evaporator at $\leqslant$80°C until crystals began to form. Sufficient water was then added to redissolve the crystals in hot solution. The solution was then set aside in the dark to crystallise. The small red needle-like crystals which formed were filtered off and dried between filter papers. A further crop of SNP was obtained from the resulting mother liquor by evaporating to dryness and extracting with a little warm methanol by stirring for 45 minutes. The solid residue, mostly sodium nitrate, was filtered off and the filtrate was treated in the same manner as previously stated and then left to crystallise. The yield of sodium nitroprusside-^{14}C ($Na_2[Fe(^{14}CN)_5NO].2H_2O$) was 1.96g, 6.58mmol, (49%),

which had a specific activity of 61 μCi mmol^{-1}. The spectrum was identical to that of a sample of AnalaR SNP; i.e. λ_{max} 394nm. Addition of the product to an alkaline solution of cysteine gave the expected[34] colour change - a positive test for the presence of SNP. Quantitative IR analysis showed the product to contain < 2% nitrate as the major impurity.

2.2.2 VISIBLE SPECTROSCOPIC ANALYSIS

Materials and Method

Hydroxocobalamin and cyanocobalamin were purchased from Sigma. SNP and KCN were of AnalaR grade. All spectra were recorded on a Pye Unicam SP8-100 spectrophotometer. Quartz cuvettes (3.0ml) were used and teflon-stoppered cuvettes were used for spectral measurements in the presence of cyanide. All experiments were carried out at 25°C and the solvent used was isotonic phosphate buffer, pH 7.2.

Isotonic buffer is a 0.02M phosphate buffer in 0.9% sodium chloride solution. This should have a pH of 7.2, with an ionic strength similar to that of whole blood. The phosphate used consists of three mole equivalents disodium hydrogen phosphate to two mole equivalents sodium dihydrogen phosphate in 0.9% sodium chloride solution.

All reactions involving cyanide were carried out in a good fume hood. Where aqueous solutions of nitroprusside were used, these were carefully protected from light. Concentrations of the H_2OCb and CNCb were 7.2×10^{-5} mol dm^{-3} in all spectra. To ensure complete reaction potassium cyanide and SNP were in limited concentrations: 3.6×10^{-5} mol dm^{-3}.

The visible absorption spectrum of aquocobalamin gives rise to a characteristic $\pi \longrightarrow \pi^*$ transition[35] of the corrin ring, a broad asymmetric shoulder at λ 529nm, $\varepsilon = 9.0 \times 10^3$ mol cm^{-1}, designated α band and its vibrational fine structure β band λ 490nm, $\varepsilon = 8.7 \times 10^3$ mol cm^{-1}, and D band λ 404nm, $\varepsilon = 3 \times 10^3$ mol cm^{-1}.

In comparison the visible spectrum of cyanocobalamin gives rise to a broad shoulder observed at λ 552nm, $\varepsilon = 8.2 \times 10^3$ mol cm^{-1}, α band; λ 524nm, $\varepsilon = 7.7 \times 10^3$ mol cm^{-1}, β band; and λ 408nm, $\varepsilon = 3.4 \times 10^3$ mol cm^{-1}, D band. All are due to $\pi \longrightarrow \pi^*$ transitions of the corrin ring system.[35]

The nitroprusside shows weak absorption in the visible region at λ 480nm due to $d_{xy} \longrightarrow \pi^*NO$ - a transition[36] to a molecular orbital containing primarily the antibonding orbital (π^*NO). At the concentrations studied this did not show any significant absorption.

2.2.3 METHOD OF EXTRACTING CYANOCOBALAMIN FROM THE REACTION MEDIUM

Materials and Method

Human blood was obtained from Dr A.R. Butler, and stored in heparinised tubes until required; experiments were carried out on the same day the blood was withdrawn. KCN-^{14}C (0.0175mCi $mmol^{-1}$) was purchased from Amersham International Ltd. Radioactive counting was carried using an EMI Ne LSC-2 liquid scintillator.

Due to spectral properties of cyanocobalamin there was massive quenching of the scintillation signal, λ 360nm. Therefore, normal counting techniques could not be employed. Instead the cyanocobalamin was collected on a porous filter disc which was suspended in a non-aqueous scintillation cocktail NE 265. This disc method was 53.5% efficient.

Before the solutions of SNP-^{14}C were added to the medium an analytical procedure for the estimation of micro quantities of cyanide[37] was carried out and the results proved negative.

The Interaction of Cyanide-^{14}C with Aquocobalamin in Blood and Extraction of Cyanocobalamin

To 10mls of whole blood and 0.015mmol of KCN-^{14}C (2.6×10^{-4}mCi) was added 0.2mmol of aquocobalamin. To this was added 0.6mmol (0.5g) of "cold"* cyanocobalamin and 30ml of AnalaR acetone to lyse the cells. This mixture was then incubated for 1 hour at 37°C. The material was then centrifuged in a bench top MSC centrifuge at 3000 r.p.m. for 30 minutes after which the supernatant was decanted off. The supernatant was then added to a 100ml separating funnel and the lipid content extracted by washing with chloroform (2 x 50ml). The resulting solution was pipetted onto a watch glass and the solvent evaporated by an IR lamp. The solid obtained was purified down a dry column of fine silica and bright red CNCb was eluted with redistilled methanol. The extracted CNCb was put down a second fine silica dry column to ensure complete purification. The CNCb was extracted from the silica by washing with redistilled methanol (3 x 100ml). The resulting solution was then concentrated on a rotary evaporator. The dark red crystalline material obtained was recrystallised from a 1:1 acetone:water mixture to constant activity as counted by the disc method mentioned previously.

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* In order to permit purification of any radioactive CNCb formed, a large excess of non-radioactive CNCb was added. This changes the activity per mole of the CNCb but still allows the formation of cyanocobalamin-^{14}C to be detected.

The Interaction of Cyanide-^{14}C and Aquocobalamin in Buffer and Extraction of Cyanocobalamin

To 10mls of isotonic buffer (pH 7.2) and 0.015mmol of KCN-^{14}C (2.6×10^{-4}mCi) was added 0.2mmol of H_2OCb and 0.6mmol (0.5g) "cold" CNCb and 15ml AnalaR acetone. The mixture was incubated for 1 hour at 37°C. After leaving for 5 hours the phosphate from the buffer had been deposited at the bottom of the flask. The resulting solution was centrifuged in a bench top centrifuge at 3000 r.p.m. for 30 minutes and the supernatant pipetted off onto a watch glass. The solvent was evaporated using an IR lamp. The resulting red crystalline material was purified twice down a fine silica dry column and eluted with redistilled methanol (3 x 100ml). The solution was then concentrated on a rotary evaporator and the dark red crystalline material was recrystallised from a 1:1 acetone:water mixture to constant activity as counted by the disc method.

The Interaction of SNP-^{14}C and Aquocobalamin in Blood and Extraction of Cyanocobalamin

To 10ml of whole blood and 0.015mmol of SNP-^{14}C (0.915 μCi) was added 0.2mmol of H_2OCb and 0.6mmol (0.5g) of "cold" CNCb. To lyse the cells 30mls of AnalaR acetone was added. The resulting mixture was protected from light and incubated for 1 hour at 37°C. The material was then centrifuged as in the previous section and the lipid content removed accordingly. The resulting solution, still protected from

light, was freeze dried to remove water and acetone. This process took approximately 3-4 hours to ensure dryness. The solid obtained was then purified down a fine silica dry column twice, protected from light at all stages. The CNCb was extracted from the silica by washing with redistilled methanol (3 x 100ml) and the solution concentrated on a rotary evaporator. The dark red crystalline material was recrystallised to constant activity as counted by the disc method.

The Interaction of SNP-^{14}C and Aquocobalamin in Buffer and Extraction of Cyanocobalamin

The same quantities of SNP-^{14}C, H_2OCb, and CNCb that were used in the experiment with blood were used here along with buffer (10mls) and AnalaR acetone (15mls). The mixture was incubated for 1 hour at 37^{o}C. After leaving for 5 hours the phosphate had deposited at the bottom of the flask. The solution was centrifuged for 30 minutes and the supernatant decanted and freeze dried. Still protected from light the solid was purified by twice passage down a fine silica dry column. The solid obtained was recrystallised to constant activity as counted by the disc method.

2.3 RESULTS AND DISCUSSION

2.3.1 VISIBLE SPECTROSCOPIC STUDIES OF THE INTERACTION OF SNP AND AQUOCOBALAMIN

All corrinoids are highly coloured and have very large extinction coefficients. This allows experimentation to be carried out with very small amounts of material.

In these studies the spectroscopic analysis has been limited to the visible region of the spectrum. Sodium nitroprusside in the visible spectrum only has a weak $d_{xy} \rightarrow \pi^{*}NO$ absorption[36] at λ 480nm, which even at high concentrations did not give any significant absorptions that interfered with the cobalamin studies.

The corrinoid ring of Vitamin B_{12} has three distictive absorption bands, α, β, D,[35] between 400-600nm (see Figure 3).

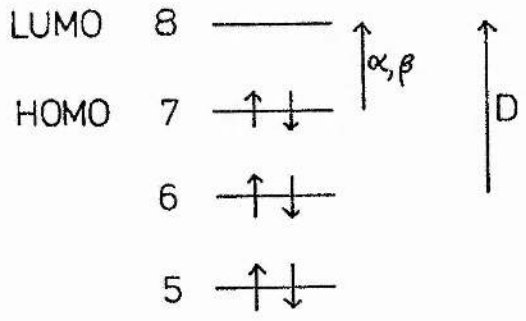

Figure 3 : Orbital Energy Pattern of the Corrin Chromophore.

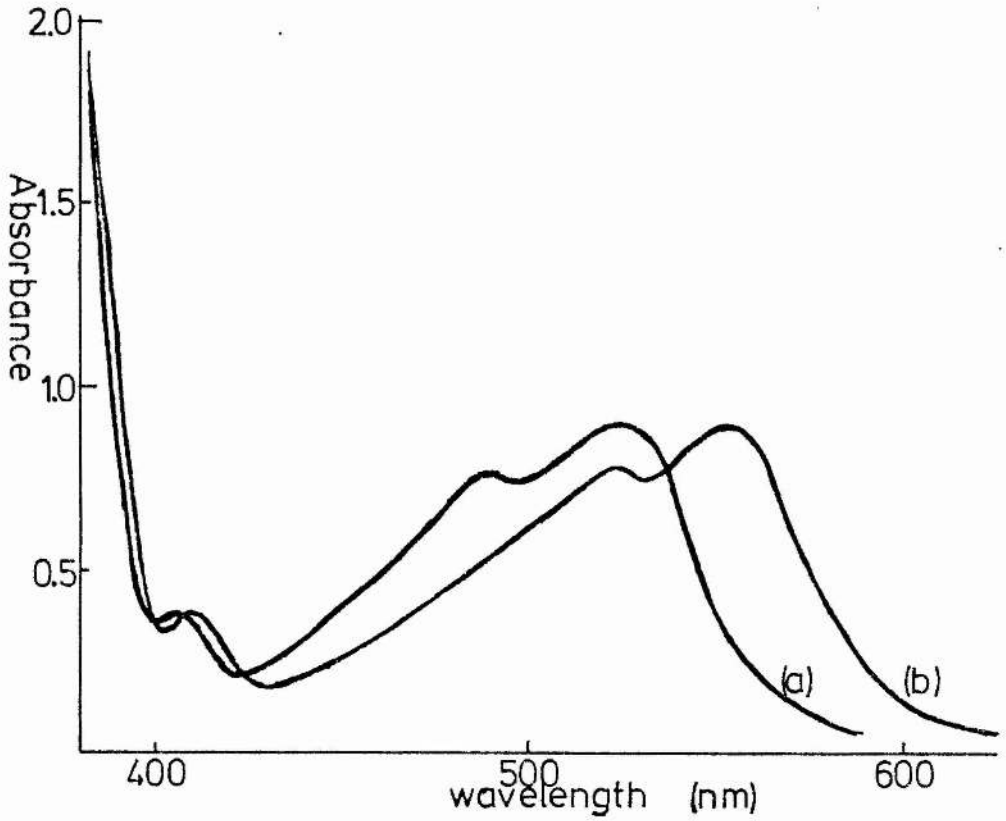

Figure 4 Plot of absorbance against wavelength.

(a) aquocobalamin 7.2×10^{-5}M

(b) cyanocobalamin 7.2×10^{-5}M

Solvent - isotonic buffer pH 7.2

Temperature 25°C

The first band is designated α and corresponds to the symmetrically forbidden transition $\pi_7 \longrightarrow \pi_8^*$:- between the highest π occupied molecular orbital of the corrin ring and the lowest unoccupied π^* molecular orbital. Although forbidden this transition is observed due to molecular polarisation.[38] The β band is the corresponding vibrational fine structure of the $\pi_7 \longrightarrow \pi_8^*$ symmetrically forbidden transition. Both α and β bands are very distinctive in cobalamins and are observed between 500 and 600nm. The third band, D, is a small band due to an antisymmetric forbidden transition $\pi_6 \longrightarrow \pi_8^*$, and is observed between 400 and 600nm.

Day[39,40] and Schrauzer[41,42] proved that the D band is particularily sensitive to electronic changes in the corrin ring system. Schrauzer[42] used iterative extended Huckel molecular orbital theory to prove that since cobalt ions interact with axial ligand π electron systems, the main effects of axial ligands may be traced back to the change of energy of π_7, the highest occupied molecular orbital, as a function of the axial ligand. Accordingly, all transitions involving π_7 (α, β) are shifted to lower energy if weakly interacting axial ligand (e.g. H_2O in aquocobalamin) is exchanged for a strongly interacting ligand (e.g. CN^- in cyanocobalamin) - see Figure 4.

Therefore, it is not surprising to note that the α band falls in energy along a series of axial ligands that closely parallels the nephelauxetic series.[39,40]

Initially SNP (3.6×10^{-5}mol dm^{-3}), H_2OCb (7.2×10^{-5}mol dm^{-3}),and CNCb (7.2×10^{-5}mol dm^{-3}) were individually monitored spectroscopically between 400 and 600nm for 12 hours to study the degree of decomposition over this period. In all three samples the absorption remains stable and no significant decomposition was apparent.

To determine how adequate a cyanide trapping agent[12] aquocobalamin is a solution of KCN (3.6×10^{-5}mol dm^{-3}) and aquocobalamin (7.2×10^{-5}mol dm^{-3}) was prepared and the visible spectrum recorded thereafter. After a period of six hours the spectrum had been converted to α band (540nm), β band (520nm),and D band (404nm). The solution was then monitored for another three hours and remained unchanged throughout that period. At those molar ratios, i.e. 2:1 aquocobalamin:KCN, if the H_2OCb was quantitatively converted to CNCb by the KCN then the resulting solution should be a combination 1:1 molar ratio of H_2OCb and CNCb. For comparison an authentic sample of a 1:1 H_2OCb:CNCb (7.2×10^{-5}mol dm^{-3}) was prepared and its spectrum recorded. This showed α band (543nm), β band (520nm), and D band (405nm). These absorptions are in reasonable agreement and show that KCN has readily been trapped by the aquocobalamin and converted to cyanocobalamin after six hours.

To study the effect of the nitroprusside anion on the visible spectrum of aquocobalamin a solution of SNP (3.6×10^{-5}mol dm^{-3}) and aquocobalamin (7.2×10^{-5}mol dm^{-3}) was monitored immediately on preparation by visible spectroscopy. The resulting spectrum α band

(532nm), β band (512nm), and D band (408nm) was obtained and remained constant over a 12 hour period. It was similar to that of H_2OCb although the bands had moved to a slightly lower energy, they remained well above both cyanocobalamin and the spectrum of the 1:1 molar ratio mixture of aquocobalamin and cyanocobalamin.

Table 1 summarises all the absorptions obtained from these initial visible spectroscopic experiments. Although helpful in showing that no SNP broke down in the presence of aquocobalamin they indicate that the nitroprusside anion itself can act as a ligand and bind to the axial position of the corrinoid ring. As a result the aquocobalamin absorption bands α and β were slightly shifted to lower energy.[40] Aquocobalamin was not converted to cyanocobalamin due to the absence of free cyanide from the decomposition of the nitroprusside anion. Chapter 4 details a further study of the effect of different molar ratios of nitroprusside:aquocobalamin on the visible spectrum of aquocobalamin.

For the moment, these initial spectral studies, although too high in concentrations for biological application, adequately answered the question of SNP decomposition under the experimental conditions described.

Table 1

Summary of visible absorption bands $\pi \rightarrow \pi^*$ of the cobalamin species.

	molar ratio	wavelength (nm)		
		α	β	D
(a) aquocobalamin		529	490	404
(b) cyanocobalamin		552	524	408
(c) aquocobalamin and KCN	2:1	540	520	404
(d) aquocobalamin and cyanocobalamin	1:1	543	520	405
(e) aquocobalamin and SNP	2:1	532	512	408

Aquocobalamin and Cyanocobalamin = 7.2×10^{-5} mol dm^{-3}

KCN and SNP = 3.6×10^{-5} mol dm^{-3}

2.3.2 ^{14}C LABELLED STUDIES OF THE INTERACTION OF $K^{14}CN$ WITH AQUOCOBALAMIN

Since radioactive labelling is a very sensitive means of analysis it can be applied to biological studies at appropriately low concentrations of material.

Initially two control experiments were carried out at biologically relevant concentrations to determine how good aquocobalamin is in buffer in comparison to whole blood at trapping free cyanide. Theoretically, if all the radioactive cyanide (0.015mmol) was trapped after an incubation period of one hour by the aquo-

cobalamin, then in both buffer and blood the specific activity of the cyanocobalamin is 3.8×10^{10} dpm mol^{-1} (where dpm is disintegrations per minute). The average of three buffer medium experiments gave the value of 7.15×10^{7} dpm mol^{-1}. In comparison three whole blood medium experiments gave the value 2.45×10^{7} dpm mol^{-1} after the same period. Relative to the theoretical specific activity there is no significant difference in the ability of aquocobalamin to trap cyanide in whole blood and buffer. Both values represent $\leqslant 0.2\%$ uptake of $K^{14}CN$ over a one hour incubation period. These results are summarised in Table 2.

Table 2

Material	Reaction Medium	Activity of Cyanocobalamin (d.p.m. mol^{-1})
$K^{14}CN$	buffer (pH 7.2)	7.15×10^{7}
$K^{14}CN$	whole blood	2.45×10^{7}
SNP-^{14}C	buffer (pH 7.2)	3.92×10^{6}
SNP-^{14}C	whole blood	4.92×10^{6}

In Chapter 3 these initial radio-labelling studies were confirmed by ^{13}C NMR spectroscopy; i.e. that the reaction of cyanide-^{13}C with aquocobalamin is slow. A freshly mixed solution, containing aquocobalamin and excess $K^{13}CN$ in isotonic buffer (pH 7.2) showed a single ^{13}C resonance assignable to $H^{13}CN$. Subsequent rerecording of the spectrum of the cyanide:aquocobalamin solution over a period of several hours showed that the resonance at 120ppm slowly decreased in intensity to be replaced by another resonance at 137.3ppm, assignable to dicyanocobalamin.[43]

These results combined with the radioactive ^{14}C labelling experiments confirm Williams[30] and Jencks[20] observations that the reaction between cyanide and aquocobalamin is slow. Moreover, they prove that whole blood does not increase the rate of conversion.

2.3.3 INVESTIGATION INTO THE INTERACTION OF ^{14}C-LABELLED SODIUM NITROPRUSSIDE AND AQUOCOBALAMIN

To investigate any decomposition, or interaction of sodium nitroprusside and aquocobalamin at biologically significant concentrations, radioactive SNP (0.015mmol) was incubated for one hour in the presence of H_2OCb in both buffer and blood. Then a method was developed to extract and purify any radioactive cyanocobalamin that may have been formed, Theoretically, if all 0.015mmol of SNP decomposed and free cyanide was trapped by the H_2OCb then the specific activity of the ^{14}C-labelled CNCb would have been 2.7×10^{10} dpm mol^{-1}. In three identical experiments using isotonic buffer (pH 7.2) the average specific activity of the extracted cyanocobalamin was 3.92×10^{6} dpm mol^{-1}. Similarly, in three identical experiments using whole blood the average specific activity of the extracted CNCb was 4.92×10^{6} dpm mol^{-1}. These results are also shown in Table 2 (see p. 29). These results, relative to the theoretical value, show no difference between the interaction of SNP and aquocobalamin in whole blood and buffer. Both values represent less than 0.02% uptake of free cyanide. It was later realised by the more descriptive technique

of ^{13}C NMR spectroscopy (see Chapter 3) combined with the visible spectroscopic studies previously discussed that sodium nitroprusside does not decompose to any significant degree, but instead binds to the aquocobalamin to form complexes which remain on the chromatographic column during the extraction for cyanocobalamin.

In conclusion these preliminary visible spectroscopic and ^{14}C radiochemical studies established that there is no difference in the rate of formation of cyanocobalamin when aquocobalamin and cyanide are incubated in either buffer (pH 7.2) or whole blood. Indeed aquocobalamin is a very bad antidote for lethal doses of cyanide poisoning.[29] Aquocobalamin is more effective in long term chronic cyanide poisoning.[44,45]

Moreover considering the medical reports, in vitro incubation of various human tissues and body fluids with nitroprusside is said to result in cyanide release. Smith and Kruszyna[5] state that blood is by far the most active biological preparation. In addition Spiegel and Kucera[4] report the quantitative release of cyanide from nitroprusside in the presence of whole blood but fail to detect cyanide in the blood of patients undergoing nitroprusside therapy when analysing aliquots taken within two minutes after infusion had terminated. In none of these above reports is there any sign as to whether precautions had been taken against photolysis of the nitroprusside which produces labile cyanide ligands.

Combined visible spectroscopic and ^{14}C labelling studies show there is no sign of decomposition of sodium nitroprusside in whole blood or isotonic buffer (pH 7.2). Instead, there is evidence that the nitroprusside ion binds to aquocobalamin: this binding is studied by the more elegant technique of ^{13}C NMR spectroscopy as described in Chapter 3.

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Chapter 3

THE INTERACTION OF CYANOFERRATES WITH VITAMIN B_{12a}

Abbreviations

SNP = Sodium Nitroprusside

H_2OCb = Aquocobalamin

CNCb = Cyanocobalamin

NP = Nitroprusside

TMS = Tetramethylsilane

TSPSA = 3-(Trimethylsilyl)propane sulphonic acid, sodium salt

CPMAS = Cross Polarisation Magic Angle Spinning

TOSS = TOtal Side band Suppression

NQS = Non-Quaternary Suppression

3.1 INTRODUCTION

Scott[1] had previously performed experiments which involved taking NMR spectra of blood samples, or of other biological molecules, which contained various substrates labelled with Carbon-13. This technique seems particularly suitable for the study of any interaction of cyanide and Vitamin B_{12a} (aquocobalamin), and the binding of sodium nitroprusside and aquocobalamin.

Although the concentrations are too high to be biologically relevant, nevertheless the technique is far more informative in both a quantitative and qualitative sense than the previous electronic spectroscopic and ^{14}C- labelling experiments described in Chapter 2. The ^{13}C NMR technique would enable any reaction to be monitored *in situ* without the necessity for further chemical manipulations, as in the lengthy ^{14}C-labelling experiments.

The natural abundance ^{13}C NMR spectra of various iron cyano complexes including nitroprusside have been obtained.[2,3] The nitroprusside ion has been shown by X-ray analysis to have C_{4v} symmetry.[4] Therefore, the nitroprusside spectrum shows two singlets in the ratio of 4:1, assigned to the four equatorial cyanides and a single axial cyanide *trans* to the NO group. The chemical shifts are 139.0 (4) and 137.0 (1) ppm.

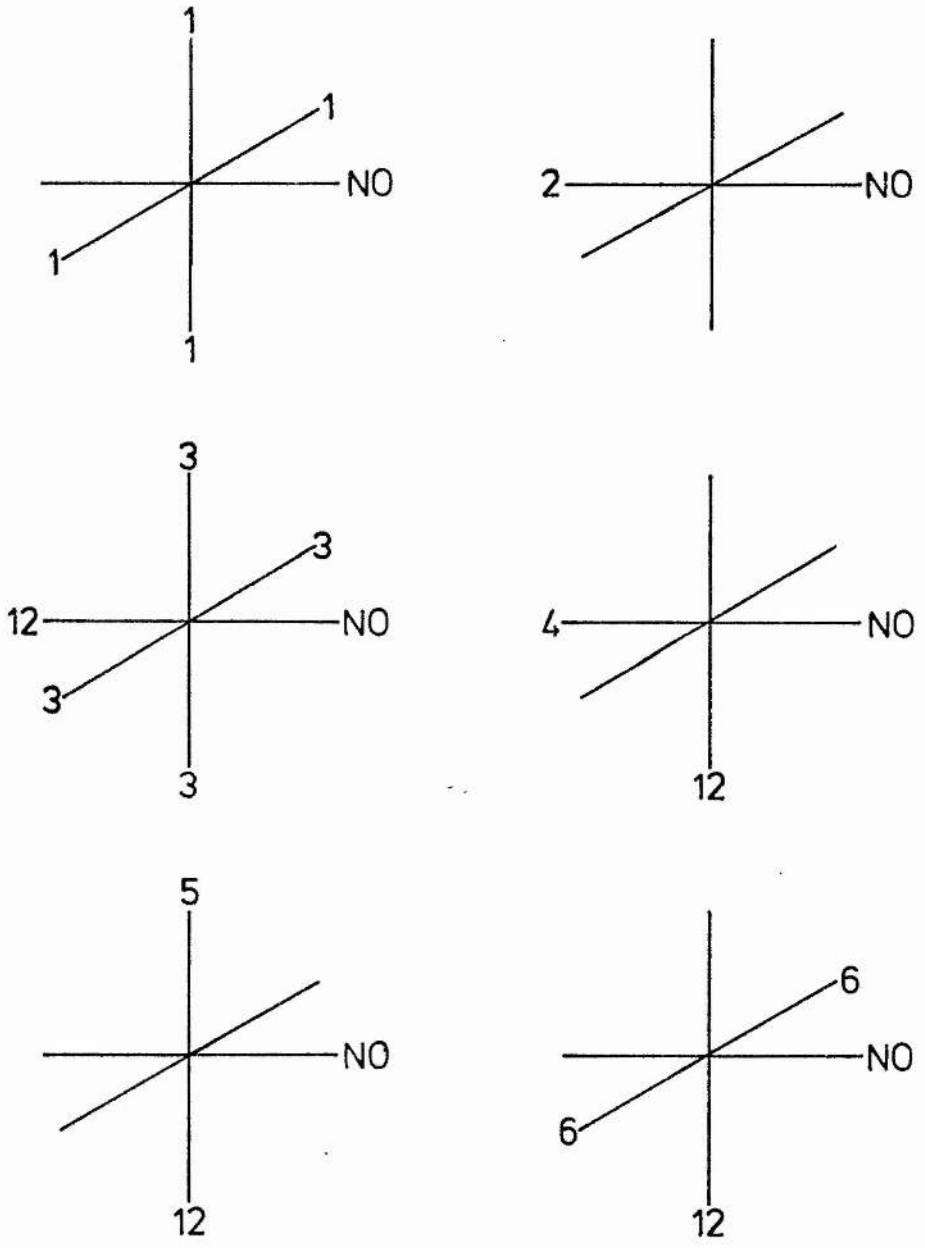


Figure 1 Non-equivalent ^{13}C environments in totally labelled and singly unlabelled nitroprusside-^{13}C.

The environments are numbered 1-6; each atom in a particular environment is indicated by the same number. "12" represents ^{12}C.

The probability of there being two ^{13}C atoms in one nitroprusside is negligible since the natural abundance of ^{13}C is so low ($\leqslant 1\%$). As a result the natural abundance spectrum obtained exhibits four ^{12}C cyanides and a single ^{13}C cyanide ligand. For the major part of this study 90% ^{13}C-labelled nitroprusside was used. Therefore the spectrum obtained becomes more complex, as has been described previously.[5] It is necessary to look in more detail into the implications of 90% ^{13}C-labelled incorporation of the nitroprusside ion so that the spectra and the splitting pattern in this present NMR study can be adequately interpreted and compared with computer spectral simulation.

Initially, consider the relative proportions of fully labelled (five ^{13}CN), singly unlabelled (four ^{13}CN), and doubly unlabelled (three ^{13}CN) species present. These proportions can be calculated from the binomial expansion $(9+1)^5$; 99% of the total can be accounted for by the first three terms of the expansion ($9^5 = 59049$, $5 \times 9^4 = 32805$, $10 \times 9^3 = 7290$); other species are insignificant. Thus 90% ^{13}C-labelled sodium nitroprusside consists of :-

(i) 59.0% $[Fe(^{13}CN)_5NO]^{2-}$,

(ii) 32.8% $[Fe(^{13}CN)_4(^{12}CN)NO]^{2-}$,

(iii) 7.3% $[Fe(^{13}CN)_3(^{12}CN)_2NO]^{2-}$.

Different isomers are obtained due to the presence of ^{12}C atoms and their position within each species. In addition there are magnetically non-equivalent ^{13}C environments within each isomer.

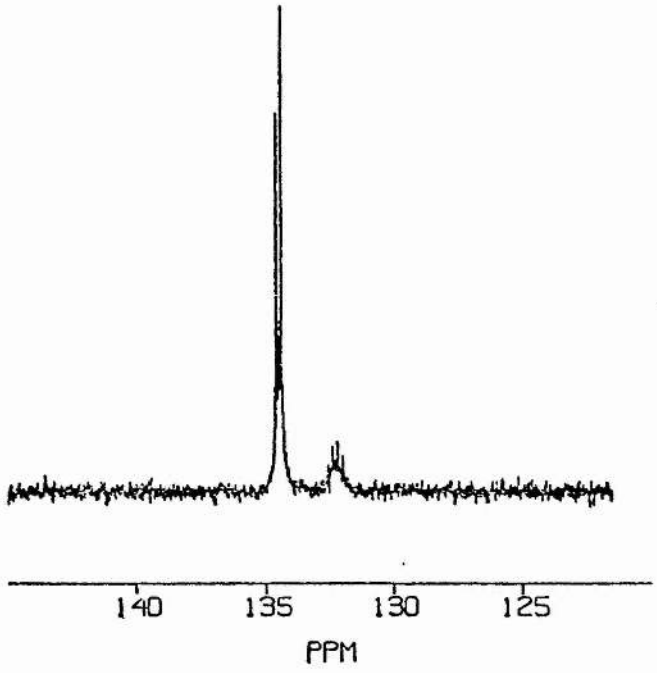


Figure 2a 90MHz ^{13}C NMR spectrum of 90% labelled SNP.

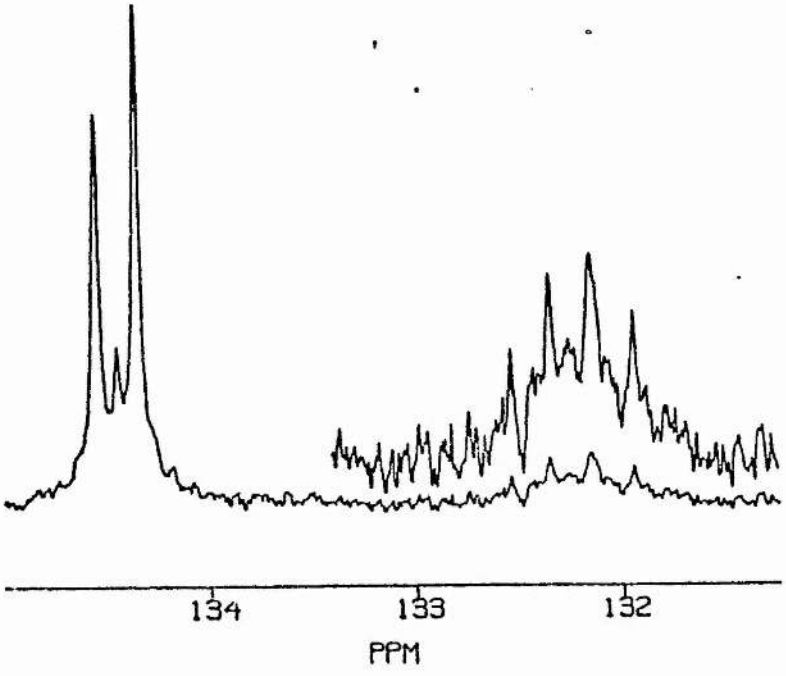


Figure 2b 90MHz ^{13}C NMR spectrum of 90% labelled SNP (expanded).

To interpret the spectra discussed in this chapter only the following species are considered. The totally ^{13}C-labelled species has only one isomer, which has two ^{13}C environments, axial (one unique ^{13}CN) and four equatorial carbons. Two isomers, in the relative ratio 4:1, are present in the singly unlabelled species, one with the ^{12}C atom in an equatorial position and the other with the ^{12}C in the axial position. In the isomer with ^{12}C equatorial there are three environments (see Figure 1);

(i) ^{13}C *trans* to NO (one ^{13}CN),

(ii) ^{13}C *cis* to NO and *trans* to ^{12}C (one ^{13}CN),

(iii) ^{13}C *cis* to NO and *cis* to ^{12}C (two ^{13}CN).

The doubly unlabelled species has three isomers and within each are a further six non-equivalent environments. Each distinct ^{13}C environment wil give rise to a separate signal in the NMR spectrum; resulting in the possibilty of coupling between ^{13}C atoms in these distinctive environments. In addition there will be a chemical shift difference due to the presence of additional ^{13}C atoms rather than ^{12}C.

Characteristic ^{13}C NMR Spectrum obtained using 90% ^{13}C-Labelled Nitroprusside

The 90MHz ^{13}C NMR spectrum of 90% labelled nitroprusside is shown in Figure 2a, and the expanded spectrum in Figure 2b. The spectrum is dominated by the totally labelled species. This gives rise to two signals; a quintet representing the axial ^{13}C split by the four equiv-

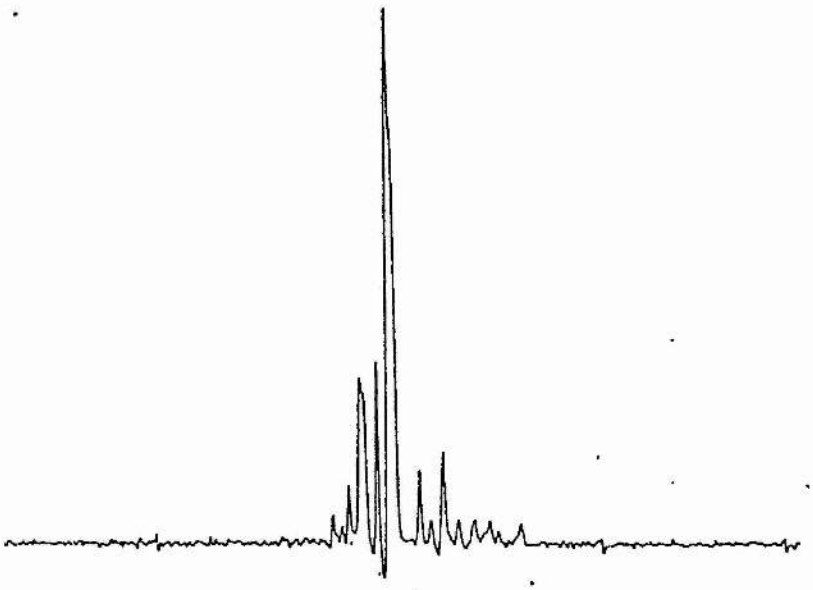


Figure 2c 20MHz ^{13}C NMR spectrum of 90% labelled SNP. Major peak at 136.6 (scale 10.4 Hz/cm).

alent equatorial ^{13}C atoms and a doublet representing the four equatorial ^{13}C split by the single axial ^{13}C. As observed in the natural abundance spectrum $\Delta\nu$ is $\sim$ 2ppm, and the coupling constant J = 18Hz. A third line is visible between the two lines of the doublet in the the expanded spectrum (Figure 2b) and this is due to the isomer of the singly unlabelled species with the ^{12}C carbon axial. In this isomer (see Figure 1) all four ^{13}C equatorial ligands are equivalent and hence give rise to a singlet spectrum. The signals due to the other axial carbons are of low intensity and are further diminished by coupling; they appear only as the broad area above which the quintet can just be discerned. Accordingly, the signals from other equatorial carbons make up the broad unresolved area at the base of the doublet.

The spectrum of 90% labelled nitroprusside recorded on a 20MHz NMR spectrometer is quite different (see Figure 2c). The discernible feature is fourteen lines, one of which is much more intense than the rest. This is a highly distorted non-first order spectrum. At 20MHz the separation of the axial and equatorial signals is 40Hz (2ppm); the coupling constant remains 18Hz and hence $\Delta\nu \gg J$. This spectral pattern is characteristic of nitroprusside.

Initially the ^{13}C-labelled NMR technique was employed to study the interaction of $Na^{13}CN$ and aquopentacyanoferrate(III), [Fe(III)-$(^{13}CN)_5H_2O$], with aquocobalamin by the 90MHz high field NMR spectrometer. Subsequently, the stability of sodium nitroprusside in the presence of aquocobalamin was studied. The apparent binding of nitroprusside and H_2OCb observed by the radiation and visible spectroscopy studies as discussed in Chapter 2 was investigated in

more depth by ^{13}C NMR. This study was extended to an analogue of both Vitamin B_{12a} and SNP using ^{59}Co NMR, and solid state ^{13}C NMR.

Finally, Extended Huckel Molecular Orbital (EHMO) calculations analysing the binding of SNP and hexacyanoferrate(II) to methylcobaloxime and to a cobalt corrin are reported.

3.2 EXPERIMENTAL

3.2.1 SOLUTION STATE NMR SPECTRA

Materials and Instruments

Sodium cyanide (90% atom ^{13}C) was purchased from MSD Isotopes Ltd. (Canada) and was used as received. Samples of $Na_4[Fe(II)(^{13}CN)_6].10H_2O$ and $Na_2[Fe(II)(^{13}CN)_5NO].2H_2O$ were prepared by Dr J. McGinnis as previously reported.[5] $Na_2[Fe(III)(^{13}CN)_5].H_2O$ was prepared by A.E. Barclay using published methods.[6] CNCb, H_2OCb, bovine serum albumin (fatty acid and globulin free), protoporphorphyrin IX (disodium salt), and poly-L-asparagine were purchased from Sigma Ltd. Samples of $CH_3Co(dmg)_2H_2O$ (dmg = dimethyl glyoxime) was prepared using published procedures.[7] All other chemicals used were of AnalaR grade.

All solutions of nitroprusside were protected from light at all times: solutions of sodium cyanide were handled in a good fume hood and all NMR sample tubes were stoppered.

NMR spectra were recorded on a Bruker WH-360 spectrometer in the FT mode and at 25°C at the Science and Engineering Research Council (S.E.R.C.) regional high field NMR service at the University of Edinburgh. The carbon resonance is at 90.56MHz in a field of 7.5T. Other ^{13}C spectra were recorded on a Varian CFT-20 instrument operating at 25°C with a carbon resonance of 20MHz in a field of 1.9T.

Experiments on the 90MHz Spectrometer

The ^{13}C spectra were recorded at 90.56MHz with TMS as an external reference using spectral widths of 12-14KHz, typically with 1000-2000 scans and with a delay of 1.38 seconds between pulses of 3 μs. The ^{59}Co spectra were recorded at 85.45MHz with $K_3[Co(CN)_6]$ as the external reference, using a spectral width of 125KHz with 14000-20000 scans and pulses of 4 μs.

All spectra involving cobalamins and those of hexacyanoferrate cobaloxime mixtures were recorded on solutions made up in 10% deuterated isotonic phosphate buffer, pH 7.2. Spectra of nitroprusside and cobaloxime mixtures, for reasons of solubility, were recorded in methanol-d_4. The concentrations varied depending upon the exact stoichiometry required, but usually the cyanoferrate concentration was 5.4×10^{-3} mol dm^{-3} (approximately 1.5%). In no case was it found necessary to use relaxation reagents. Experimental uncertainties in spectral parameters are $\pm$ 0.1ppm in chemical shifts and $\pm$ 0.2Hz in coupling constants.

Experiments on the 20MHz Spectrometer

A series of preliminary experiments was carried out generally at concentrations of 0.05mol dm^{-3} of 90% ^{13}C-labelled cyanoferrates in 10% D_2O isotonic buffer pH 7.2 using TSPSA as the external reference. The number of scans was typically 2000-4000 with a pulse width of 7 μs and a pulse delay of 0 seconds.

Table 1

Quantities of reagents used in the NMR experiments, the numbers refer to the figure which shows that spectrum. All values are stated as mol $x10^5$.

Figure	SNP	H_2OCb	$[Fe(CN)_6]^{4-}$	$MeH_2OCo(dmg)_2$
3	1.08	11.20		
4	1.08	1.08		
5	1.08	1.62		
6a	1.08	2.16		
6b	1.62	2.16		
9		1.08	1.08	
10		2.16	1.08	
11		5.40	1.08	
12	1.08			2.16
13	1.08			5.40
14a				62.00
14b	125.00			62.00
15			1.08	1.08
16			1.08	6.50

Natural abundance ^{13}C NMR spectra of the cyanoferrates were generally run in 1mol dm^{-3} 10% D_2O phosphate buffer (pH 7.2) solutions using TSPSA as the external reference. Most spectra required 13000-15000 scans with a pulse width of 7 μs and a pulse delay of 0 seconds.

Computer Spectral Simulation

All spectral assignments were checked by spectral simulation using the "Simeq" spin simulation program in conjunction with the hardware computer facilities of the Varian CFT-20 NMR spectrometer. In the case of 90% ^{13}C-labelled SNP this involved the summation of the spectra due to $[Fe(^{13}CN)_5NO]^{2-}$ and both isomers of $[Fe(^{13}CN)_4(^{12}CN)NO]^{2-}$.

3.2.2 SOLID STATE NMR STUDIES

All ^{13}C solid state NMR spectra were recorded on a Varian VXR 300MHz spectrometer with solid state attachment and wide bore Oxford magnet at the S.E.R.C. solid state NMR service at the University of Durham. The carbon spectra were recorded at 75.43MHz relative to external TMS. All spectra were run at 25°C and obtained with a high-power proton decoupler, typically with 1000-10000 transients, spectral widths of 50000Hz and a delay of 2.0s between pulses of 6μs.

^{13}C solid state NMR spectra for samples of both 90% ^{13}C-labelled sodium hexacyanoferrate and aquocobalamin were recorded using Cross Polarisation Magic Angle Spinning (CPMAS).[8]

A sample of 1:1 mole ratio ^{13}C-labelled sodium hexacyanoferrate and aquocobalamin was prepared by dissolving 2.06 x 10^{-4} moles of both hexacyanoferrate and aquocobalamin in a minimum volume of isotonic buffer pH 7.2. The resulting solution was incubated at 25°C for one hour and then the solvent removed by freeze drying. The ^{13}C solid state NMR spectrum of the 1:1 sodium hexacyanoferrate:H_2OCb was initially run using CPMAS. To simplify interpretation a spectrum was run using CPMAS and TOtal Side band Suppression (TOSS) mode.[8] To further simplify the solid state spectrum of the sample a spectrum using CPMAS and Non-Quaternary Suppression (NQS) mode[8] was recorded.

3.2.3 MOLECULAR COMPUTER GRAPHICS

All atomic parameters and structural data were obtained from the Cambridge Crystal Structure Search and Retrieval Database (Daresbury) provided by the S.E.R.C.

The program ChemX in conjunction with the Vax 11/785 computer was employed for subsequent molecular modelling. Graphical output was displayed on a Tektronics 4109 colour terminal and molecular models photographed directly from the screen, or plotted out using a Tektronics 4695 colour ink spray plotter. I am indebted to the National Foundation for Cancer Research (NFCR) (U.S.A.) group at St Andrews University for their guidance and use of Tektronics hardware.

3.2.4 EXTENDED HUCKEL MOLECULAR ORBITAL CALCULATIONS

All molecular orbital calculations were made with the extended Huckel method[9,10] using published atomic parameters.[11] Structural data were taken from X-ray studies.[4,12,13] Electrostatic potentials were calculated and plotted[14] using the net atom charges taken from the extended Huckel calculations. I thank Dr C. Glidewell for carrying out the EHMO calculations which are reported herein.

3.3 RESULTS AND DISCUSSION

3.3.1 REACTIONS OF CYANIDE AND AQUOPENTACYANOFERRATE(II) WITH COBALAMINS: MONITORED BY ^{13}C-LABELLED NMR SPECTROSCOPY

As outlined in Chapter 2 there appears to be a large number of conflicting reports about the rate of reaction of aquocobalamin with cyanide in a biological medium; whole blood and isotonic buffer. Previous preliminary work using visible electronic spectroscopy and ^{14}C radioactive labelling experiments indicated that in both buffer and whole blood the reaction, although effectively irreversible, is very slow. These results are in contradiction to many reports[15-17] in the medical literature recommending H_2OCb as an excellent antidote for potentially lethal doses of cyanide poisoning. Although these preliminary studies gave consistent results at biologically relevant concentrations, the more descriptive technique of ^{13}C NMR spectroscopy would enable any reaction to be monitored *in situ* without the necessity for further chemical manipulation.

Since there was no difference in the rate of reaction using either whole blood or isotonic buffer in the ^{14}C-labelling experiments for convenience the NMR spectra were run in isotonic phosphate buffer (pH 7.2).

Initially the spectrum of sodium cyanide-^{13}C (0.028mol dm^{-3}) was recorded in a high field spectrometer; this gave a resonance at 120.9ppm arising from undissociated hydrogen cyanide-^{13}C. To this solution H_2OCb (5.6×10^{-2}mol dm^{-3}) was added and scans were accumulated for one hour and then the spectrum recorded. This showed only a single peak at 120.9ppm attributable to $H^{13}CN$. On this time scale there was no detectable reaction of the cyanide with two-fold molar excess of H_2OCb.

When a freshly mixed solution containing sodium cyanide-^{13}C (0.1mol dm^{-3}) and H_2OCb (0.05mol dm^{-3}), (two-fold molar excess of $Na^{13}CN$) was used only a single ^{13}C resonance was obtained, again at 120.9ppm. Only after several hours did the spectrum show a decrease in intensity of the HCN resonance which was replaced by a new peak at 137.3ppm assignable to dicyanocobalamin.[18] The two chemically distinct cyanide ligands of the dicyanocobalamin did not, at 90MHz, show any detectable chemical shift difference, whereas the cyanide resonance for the two isomers, α and β, of aquocyanocobinamide exhibited a chemical shift difference of 1.1ppm.

The initial product of sodium nitroprusside photolysis is aquopentacyanoferrate(III),[19] this is a labile d^5 paramagnetic species. When the $[Fe(III)(^{13}CN)_5H_2O]^{2-}$ anion was added to two-fold molar excess of H_2OCb no cyanide transfer to form the cyano- or dicyanocobalamin was observed. To ensure that the failure to observe any signal in the region of 137ppm cannot be ascribed to the paramagnetism of the aquopentacyanoferrate(III), a set of control experiments were

carried out. Spectra were recorded on solutions of the diamagnetic cyanometalates, nitroprusside $[Fe(II)(CN)_5NO]^{2-}$, and hexacyanoferrate(II) $[Fe(II)(CN)_6]^{4-}$, which were titrated against the paramagnetic aquopentacyanoferrate $[Fe(III)(CN)_5H_2O]^{2-}$. These showed that the spectra of the diamagnetic species were not perturbed in any way by the presence of a paramagnetic ion, at least not at the concentrations studied (typically 5.4×10^{-3} mol dm^{-3}).

These ^{13}C high field NMR experiments combined with initial ^{14}C-labelling and visible spectroscopy studies prove that, whether in whole blood or buffer, the reaction of aquopentacyanoferrate(III) and cyanide at physiological pH is slow.

These results are consistent with those of Williams[20] who reported for the formation of cyanocobalamin from aquocobalamin a value of $K > 10^{12}$ but found, at concentrations of 2.5×10^{-5} mol dm^{-3} and pH 4.75, a half-life for cyanocobalamin formation of some four and a half hours. A subsequent determination[21] gave $K = 1.2 \times 10^{14}$. Jencks[22] has studied in great detail the reactions of cyanide and hydrogen cyanide with H_2OCb and CNCb to form mono- and di-cyano derivatives, whereas Conn[23] has found that the conjugate base, hydroxocobalamin, does not react at all.

3.3.2 REACTIONS OF NITROPRUSSIDE AND AQUOCOBALAMIN STUDIED BY ^{13}C NMR SPECTROSCOPY

To determine any long term decomposition of the nitroprusside ion, (Photograph 1) a solution of ^{13}C-labelled SNP (0.0054mol dm^{-3}) was incubated for one week with aquocobalamin (0.056mol dm^{-3}) at 37°C in isotonic buffer pH 7.2 (10% D_2O). The spectrum obtained exhibited no resonance for mono- or dicyanocobalamin-^{13}C at 137ppm. Although the conversion of aquocobalamin to cyanocobalamin is slow at physiological pH, if the nitroprusside ion had decomposed to give free cyanide then after a period of one week there would have been a sufficient amount of ^{13}C-labelled cyanocobalamin to obtain a signal at 137ppm. Therefore, it can be concluded that over a period of one week, with incubation at 37°C, there is no decomposition of the nitroprusside ion. Since *in vitro* ^{14}C experiments (Chapter 2) established no significant difference in the rate of SNP in whole blood or buffer, then the present NMR results in buffer are comparable with incubating SNP with aquocobalamin in whole blood for the same period.

Combined ^{13}C-labelling NMR studies in isotonic buffer and the more sensitive ^{14}C-labelled experiments in both buffer and whole blood strongly refute medical reports[24-27] indicating cyanide release as a result of *in vitro* incubation of various human body tissues and body fluids, especially whole blood[28,29] with sodium nitroprusside.

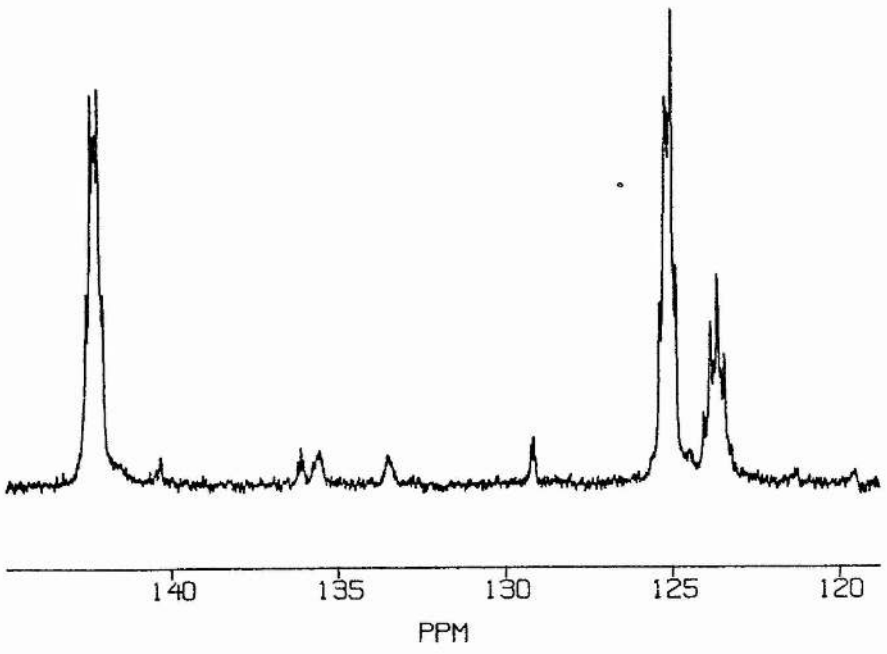


Figure 3a 90MHz ^{13}C NMR spectrum of a 10:1 molar ratio of H_2OCb and 90% labelled SNP in isotonic buffer (10% D_2O) pH 7.2.

There are confusing results in the medical literature about the rate of supposed release of cyanide from the *in vitro* incubation of SNP with whole blood. Speigel and Kucera[30] state that cyanide release is rapid without actually quoting any data relevant to the rate beyond the observation of 100% decomposition over the course of a two hour diffusion during analysis. Vesey *et al*[24,31(a)] report the release of cyanide to be slow with 50% decomposition in twenty minutes and over 90% decomposition in two hours. In a later paper[29] the same workers report only 46% decomposition of nitroprusside after three hours *in vitro* incubation with whole blood.

The non-intrusive NMR experiment of incubating ^{13}C-labelled SNP in whole blood for fourteen hours investigated previously in this department[31] coupled with the present non-intrusive ^{13}C NMR experiment incubating SNP-^{13}C with aquocobalamin in isotonic buffer for one week prior to recording the spectrum has established no *in vitro* decomposition of SNP in the presence of whole blood or buffer. Those medical reports of cyanide release are based on results obtained by intrusive methods of analysis (see Chapter 1, p. 4) and mention no precautions taken to keep the nitroprusside solutions away from light.

When ^{13}C-labelled nitroprusside was incubated for one week with aquocobalamin at 37°C in isotonic buffer as discussed previously the spectrum obtained exhibited no sign of a resonance at 137ppm due to CNCb or the typical AX_4 nitroprusside spectrum as characterised by δ_A = 132.4, δ_X = 134.4, and J_{AX} = 17.7Hz. Instead a completely new

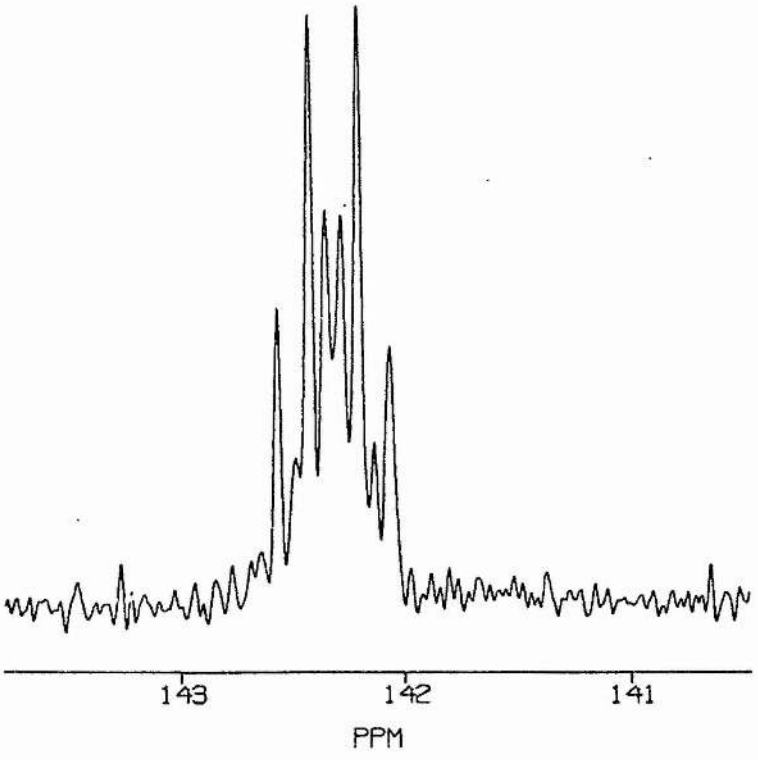
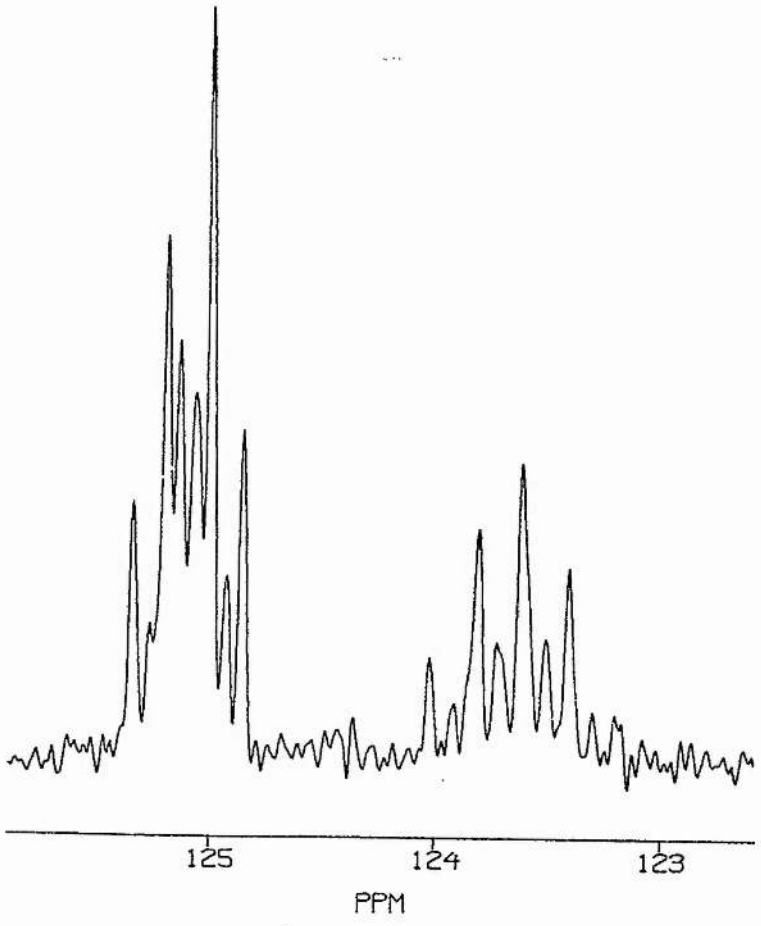


Figure 3b and 3c 90MHz ^{13}C NMR line narrowed spectrum of a 10:1 molar ratio of H_2OCb and 90% labelled SNP in isotonic buffer (10% D_2O) pH 7.2.



AM_2X_2 type spectrum was obtained; δ_A = 123.6, δ_M = 125.1, δ_X = 142.3, $J_{AM} = J_{AX}$ = 18.6Hz, and J_{MX} = 13.0Hz. When exactly the same solution was freshly prepared with a molar ratio of 10:1 H_2OCb to SNP-^{13}C and the spectrum recorded immediately an identical AM_2X_2 type spectrum was obtained (see Figure 3a,b,c,). These spectra indicate that SNP binds to aquocobalamin immediately, when the molar ratio of H_2OCb to SNP is 10:1, and that the resulting complex remains stable for at least one week at 37°C (in the absence of light). To determine whether the presence of the cobalt in Vitamin B_{12a} (see Photograph 2) is essential for this binding phenomena, or just the corrin ring system with several of its side amide functional groups, several control NMR experiments were carried out on the 20MHz spectrometer. Initially natural abundance ^{13}C NMR spectra were run on several solutions containing nitroprusside (1mol dm^{-3}) and saturated isotonic buffer (10% D_2O) solutions of urea, bovine serum albumin, malonamide, and poly-L-asparagine. All three solutions exhibited no perturbation of the typical AX_4 spectrum of nitroprusside which might result from binding to these compounds, after incubation periods of up to 14 hours at 37°C . When a corrin ring system, protoporphyrin IX, itself was incubated under the same conditions and timescale with nitroprusside, the resulting ^{13}C spectrum again showed no chemical shift change due to binding. A 90MHz ^{13}C NMR spectrum was then recorded on a solution of SNP-^{13}C and CNCb in isotonic buffer in the molar ratio 1:10 SNP:CNCb. This gave rise to the typical nitroprusside AX_4 spectrum. All these combined NMR spectra indirectly prove that if the corrin ring is not involved in the binding of the nitroprusside it must be the cobalt metal atom. In the case of cyanocobalamin the cyanide is already bound irreversibly to the cobalt, therefore no binding of the

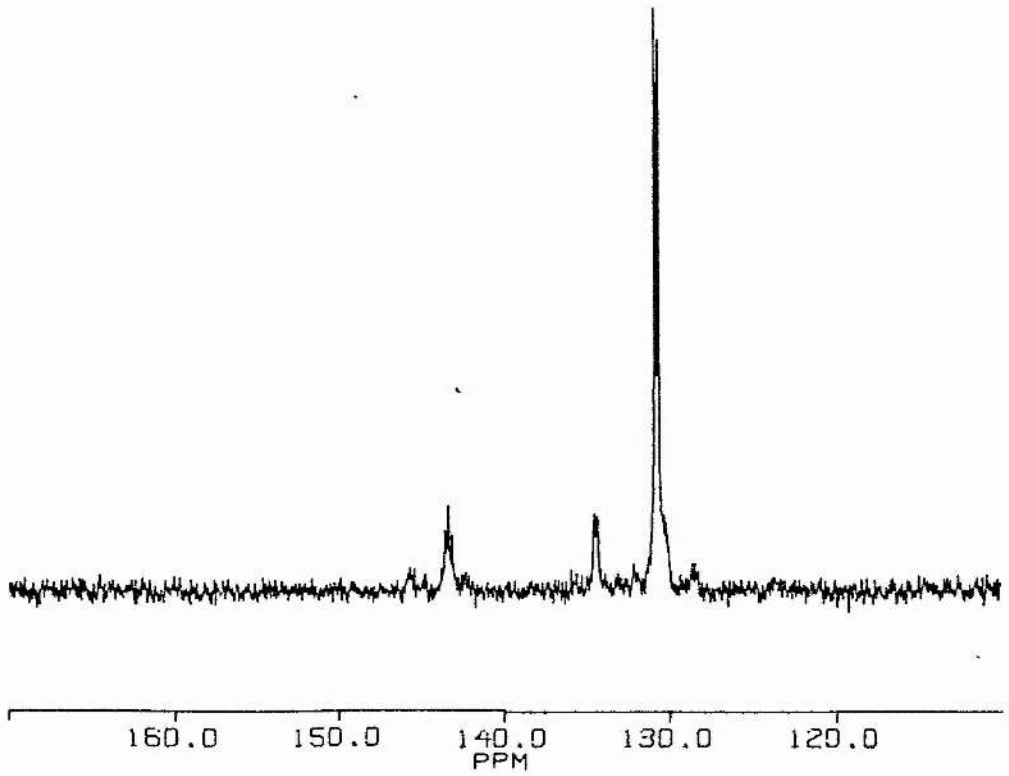


Figure 4 90MHZ ^{13}C NMR spectrum of a 1:1 molar ratio of H_2OCb and 90% labelled SNP in buffer pH 7.2.

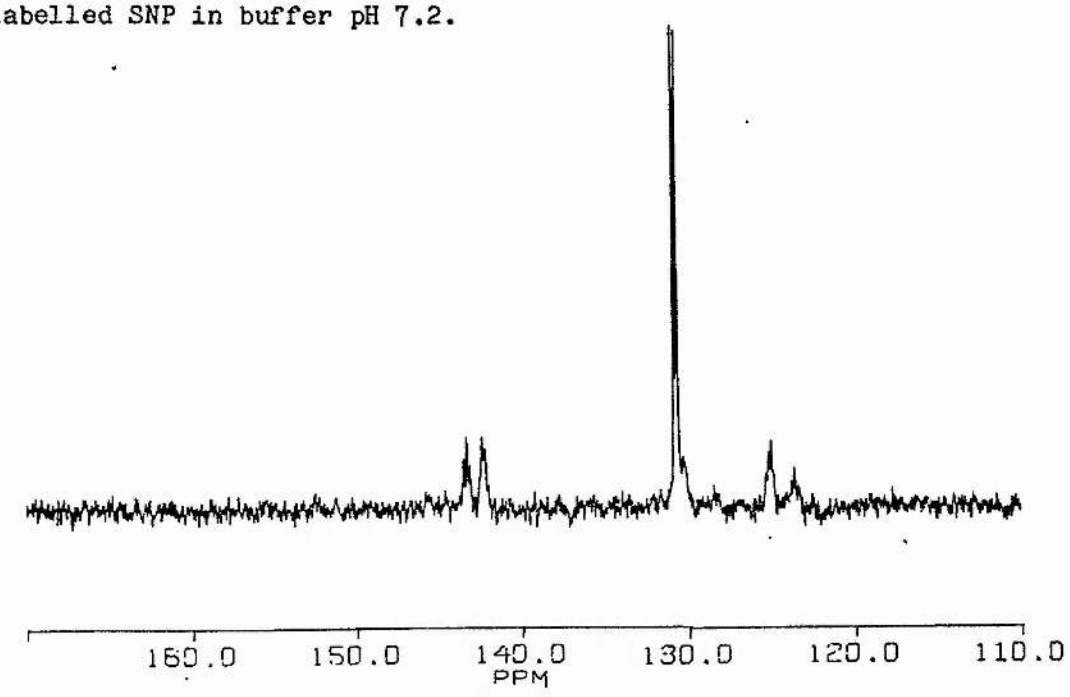


Figure 5 90MHz ^{13}C NMR spectrum of a 1.5:1 molar ratio of H_2OCb and 90% labelled SNP in buffer pH 7.2.

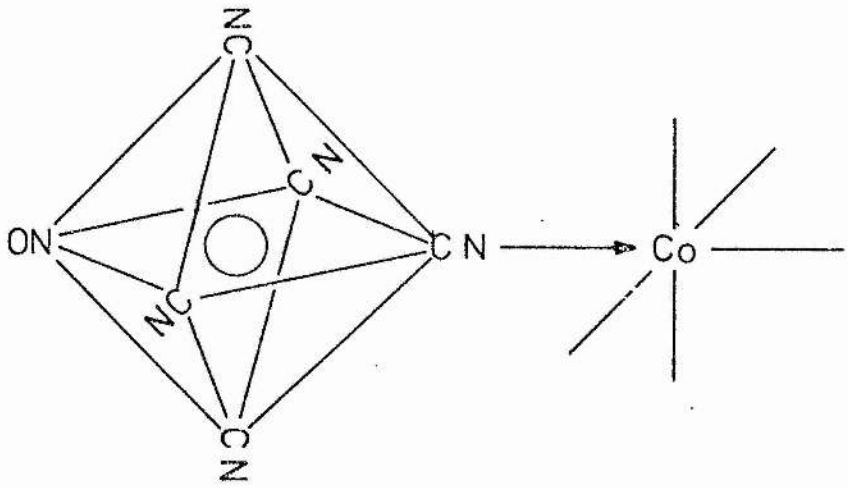
nitroprusside ion can take place; a normal spectrum of nitroprusside was obtained. No direct evidence in this instance could be obtained by employing ^{59}Co NMR since no ^{59}Co resonances in solutions of either aquocobalamin or of cyanocobalamin were detected. This is not surprising since although ^{59}Co has I = 7/2, and is 100% abundant, it has a quadrupole moment of $0.4 \times 10^{-28} cm^2$; a quadrupole moment of this intermediate magnitude makes ^{59}Co linewidths sensitive to electrical field gradients at the cobalt and therefore the symmetry about the cobalt atom.[33,34] The structure of most cobalt(III) compounds is based on an octahedral array of six ligands surrounding the cobalt; structures having lower symmetry result in lower electric field gradients and hence vast degrees of line broadening. For neither CNCb nor H_2OCb has any spectrum been obtained because of the low symmetry around the cobalt atom. The peaks may be so broad that they cannot be distinguished from the base line.

To determine more information about the binding of nitroprusside to aquocobalamin a set of NMR spectra were run on solutions of varying stoichiometries of ^{13}C-labelled nitroprusside (5.4×10^{-3} mol dm^{-3}) and aquocobalamin in 10% D_2O isotonic buffer.

Initially an equimolar quantity of aquocobalamin was added to nitroprusside and the characteristic AX_4 SNP spectrum (δ_A = 132.4, δ_X = 134.4, and J_{AX} = 17.7Hz) was rapidly and completely converted to a quite different AX_4 spectrum characterised by δ_A = 143.3, δ_X = 130.7, and J_{AX} = 18.9Hz (see Figure 4). When a second molar equivalent of SNP was added, the resulting spectrum was the summation of the two AX_4 spectra. On the NMR time scale there was no fast exchange

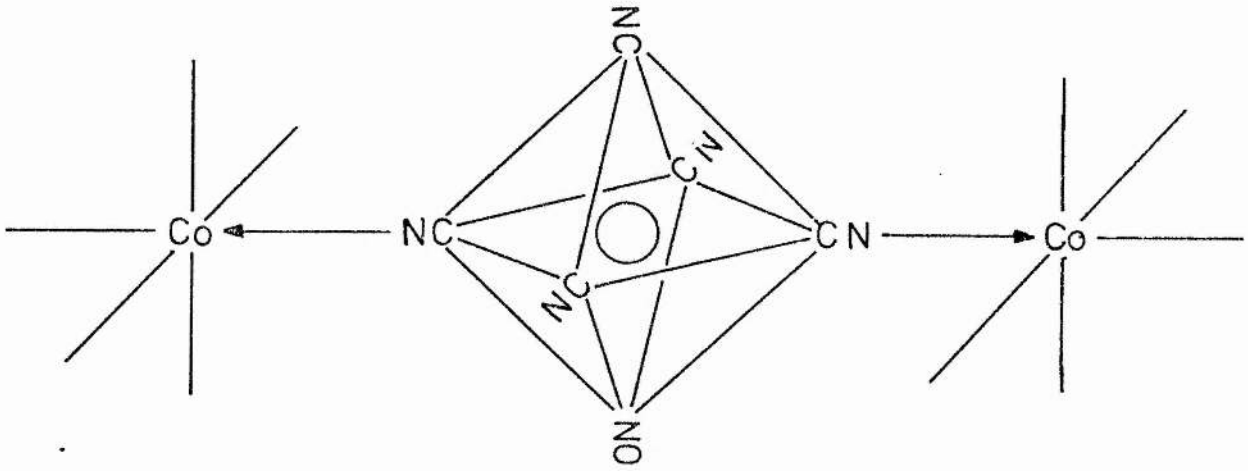
between the two pentacyanoferrate species. On closer study of the spectrum it was noted that the addition of aquocobalamin to nitroprusside at equimolar concentrations results in the equatorial resonance (δ_X) moving to lower frequency by about 4ppm, while the axial resonance (δ_A) moved to higher frequency by 11ppm. These changes are comparable with those observed[35,36] for the carbonyl resonances of polynuclear metal carbonyl species. In these species the resonance of the carbonyl ligand that is co-ordinated at the oxygen by the Lewis acid moves to much higher frequency, while the remaining carbonyl resonances generally moved to slightly lower frequency. The new AX_4 type spectrum can be assigned to nitroprusside bound to Vitamin B_{12}, where the axial cyano ligand of the nitroprusside is co-ordinated via nitrogen to the cobalt(III) of the cobalamin, Species 1 (see Photograph 3). When nitroprusside is in molar excess over aquocobalamin, on the NMR time scale there is no fast exchange between free and bound nitroprusside.

Species 1



The initial AM_2X_2 spectrum (Figure 3) was obtained as a result of SNP with aquocobalamin in a ten-fold molar excess can be explained by Species 2 (Photographs 4 and 5).

Species 2



The M and X resonances are those of the two trans pairs of equatorial cyano ligands in the new Species 2, while the A resonance is that of the unique axial ligand. The two co-ordinated cyano ligands of Species 2 are those that show the high frequency shift of 8ppm (unbound δ_X = 134.4ppm, bound δ_X = 142.3ppm). The other cyano ligands all exhibited a low frequency shift of ca 9ppm for both axial and equatorial ligands (unbound δ_A = 132.4, δ_M = 134.4; bound δ_A = 123.6, δ_M = 125.1ppm).

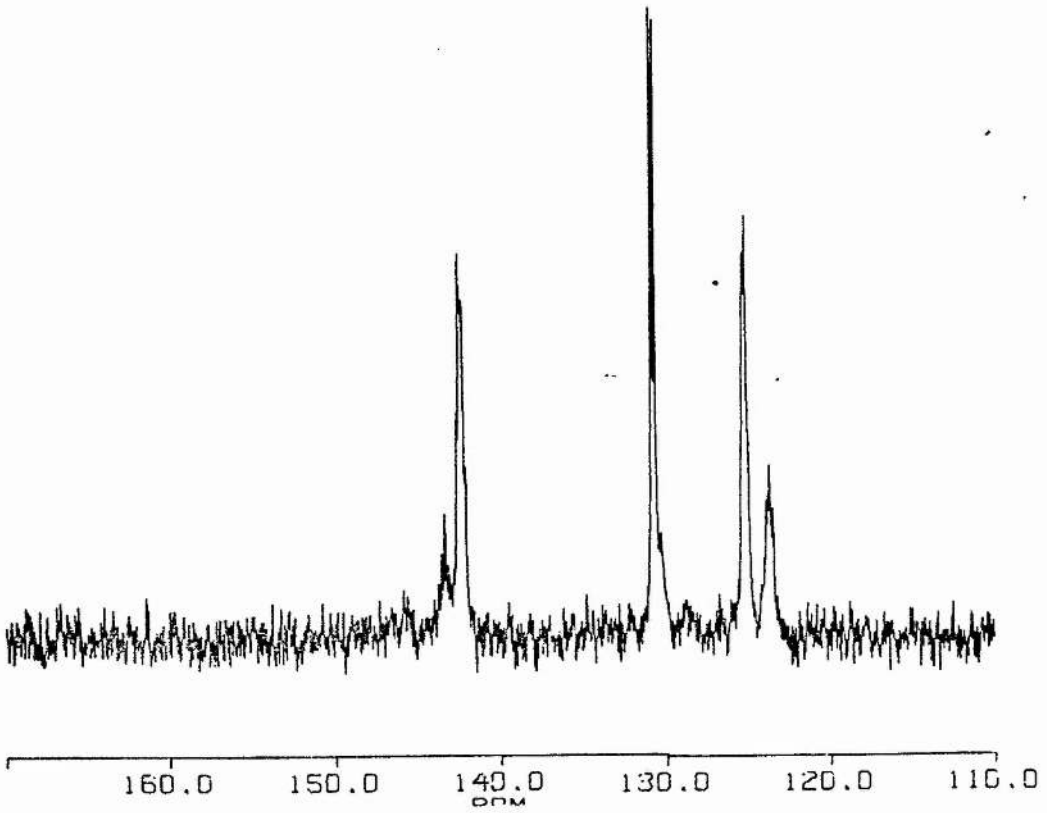


Figure 6a 90MHz ^{13}C NMR spectrum of a 2:1 molar ratio of H_2OCb and 90% labelled SNP in buffer pH 7.2.

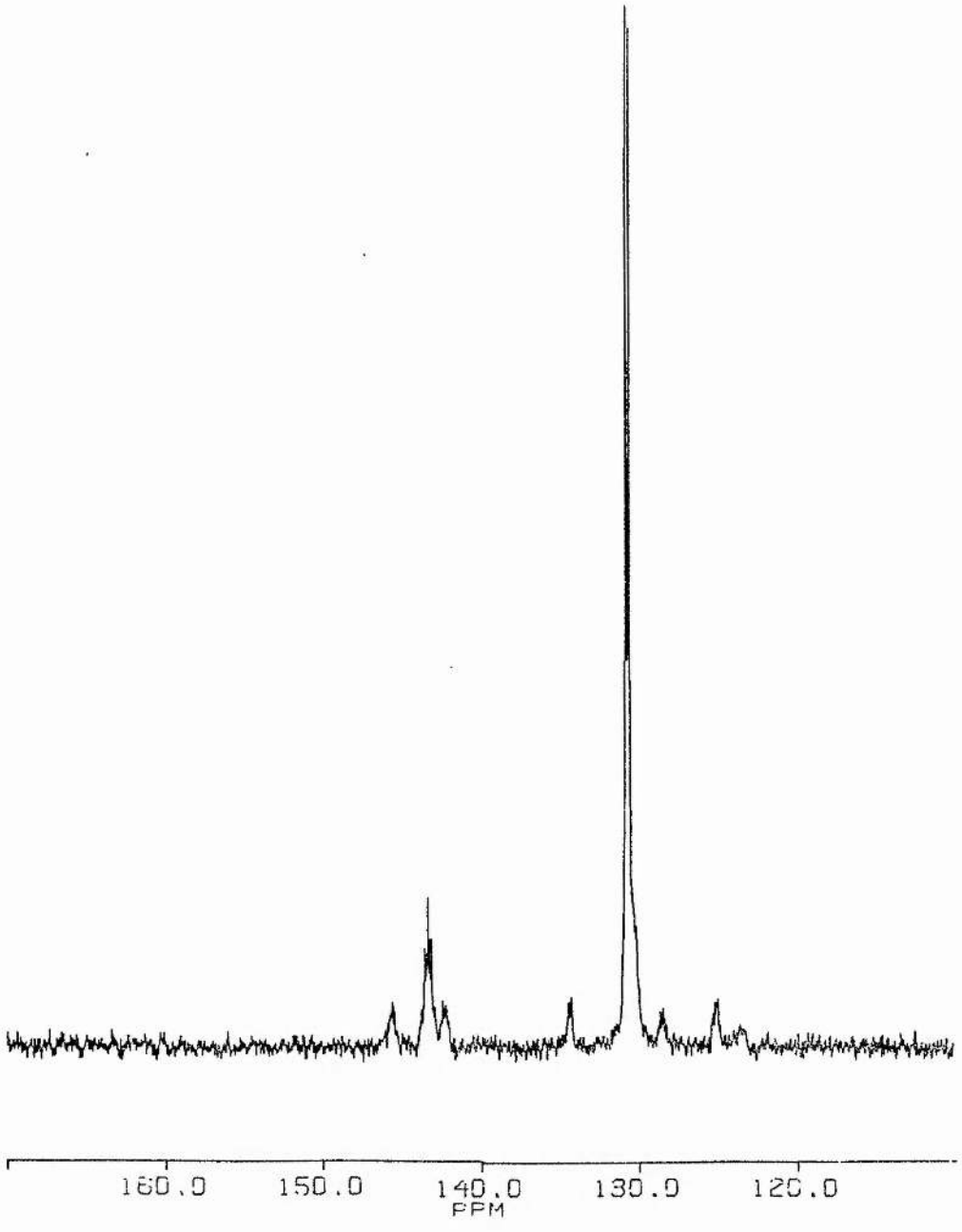


Figure 6b 90MHz ^{13}C NMR spectrum of a 2:1.5 molar ratio of H_2OCb and 90% labelled SNP in buffer pH 7.2.

When an additional one half molar equivalent of aquocobalamin was added to Species 1 (Figure 4), the spectrum was replaced by the summation of the spectra of Species 1 and 2 (Figure 5). This indicates that there was no fast exchange between the two species. When approximately a one half molar equivalent of nitroprusside was added to a solution of Species 2 (Figure 6a), virtually all of Species 2 was converted back to Species 1 within a few minutes (Figure 6b). It is possible to observe all three species ($[Fe(^{13}CN)_5NO]^{2-}$ and its 1:1 and 1:2 adducts with cobalamin) independent of one another by adjusting the relative concentrations.

In comparison with the line width observed for $[Fe(CN)_5NO]^{2-}$, the new spectra indicate no perceptible line width broadening. Hence an approximate upper limit on the rate of any exchange processes involving $[Fe(CN)_5NO]^{2-}$ and Species 1 and 2 is $1s^{-1}$. Nevertheless, the ready conversion of Species 1 to 2 by addition of aquocobalamin and the reverse transformation by the addition of further amounts of nitroprusside indicate that the equilibria are reasonably mobile.

All spectra were confirmed by a NMR spin simulation program "Simeq". This program works by producing a Hamiltonian for the desired spin system, using magnetic equivalence factoring to reduce the Hamiltonian matrix to manageable proportions. The advantage of "Simeq" is its provision for the spectra of three totally independent spin systems to be added in the appropriate ratios. In the case of the observed ^{13}C spectrum of the 1:2 90% nitroprusside-^{13}C:H_2OCb system a characteristic AM_2X_2 type spectrum was initially subjected to

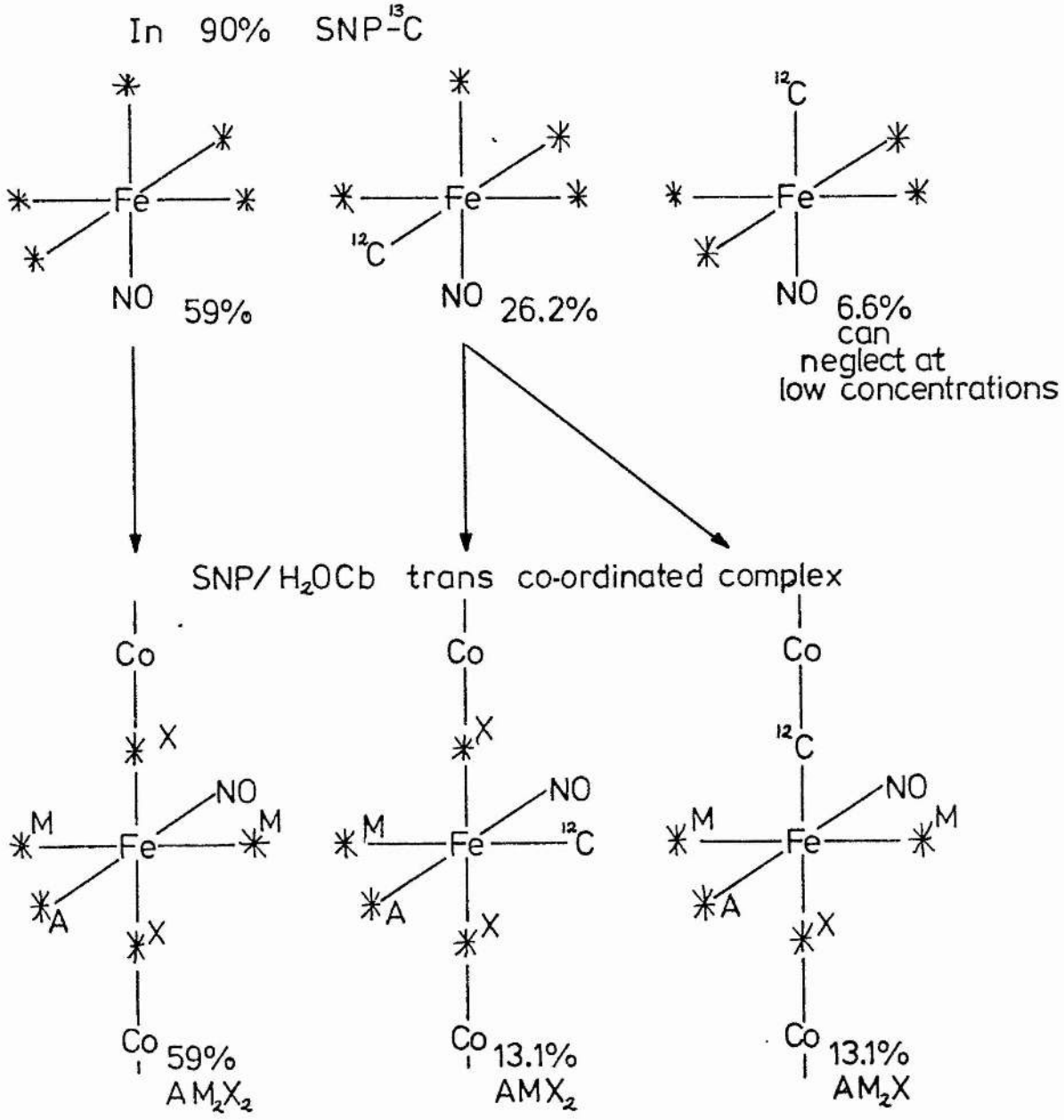


Figure 7 90% enrichment of ^{13}C in a 1:2 adduct (Species 2) with SNP and H_2OCb. In each of the three systems above the coupling constant and splitting pattern was considered as shown in Table 2.

spin simulation for three independent systems (Figure 7 and Table 2).

Table 2

Coupling constants and splitting pattern of the three independent systems.

	Spectrum Type	Coupling Constants (J/Hz)
	AM_2X_2	
$J_{AM} = J_{AX}$	A : quintet	18.6
	M : double triplet	J_1 18.6, J_2 13.0
	X : double triplet	J_1 18.6, J_2 13.0
	AMX_2	
$J_{AM} = J_{AX}$	A : quartet	18.6
	M : double triplet	J_1 18.6, J_2 13.0
	X : double triplet	J_1 18.6. J_2 13.0
	AM_2X	
$J_{AM} = J_{AX}$	A : quartet	18.6
	M : double triplet	J_1 18.6, J_2 13.0
	X : double triplet	J_1 18.6, J_2 13.0

These three independent systems gave the correct spectra. Then the three systems AM_2X_2, AMX_2, and AM_2X were combined in the correct intensity ratio 59%, 13.1%, and 13.1% respectively. The complete spin simulation was successful and gave the correct shape and line sequence system (see Figure 8 and Table 3).

TABLE 3 Spectral parameters for complexes of $[Fe(CN)_5NO]^{2-}$ = NP and $[Fe(CN)_6]^{4-}$ with aquocobalamin (H_2O.Cb) and with $CH_3Co(DH)_2H_2O$ (H_2O.Cx)

Complex	Spectral type	$\delta(^{13}C)$/ppm				Coupling constants/Hz		
		A	M	X	mean	J(AX)	J(AM)	J(MX)
$[Fe(CN)_5NO]^{2-}$(NP)	AX_4	132.4	-	134.4	134.0	17.7	—	—
NP.Cb	AX_4	143.3	-	130.7	133.2	18.9	-	-
NP.$(Cb)_2$	AM_2X_2	123.6	125.1	142.3	131.7	18.6	18.6	13.0
$[Fe(CN)_6]^{4-}$	A_6	177.7	-	-	177.7	-	-	-
$[Fe(CN)_6]^{4-}$.Cb		174.1	-	-	174.1	-	-	-
$[Fe(CN)_6]^{4-}$.$(Cb)_2$	A_2X_4 [b]	181.5	-	169.2	173.3	b	-	-
$[Fe(CN)_5NO]^{2-}$ [c]	AX_4	128.2	-	129.7	129.4	18.3	-	-
NP.Cx [c]	AX_4	126,6	-	128.5	128.1	19.2	-	-
$[Fe(CN)_6]^{4-}$.Cx		177.1	-	-	177.1	-	-	-
$[Fe(CN)_6]^{4-}$.$(Cx)_2$	A_2X_4 [b]	178.5	-	173.9	174.8	b	-	-

b No couplings resolved.

c Measured in methanol; all others in phosphate buffer.

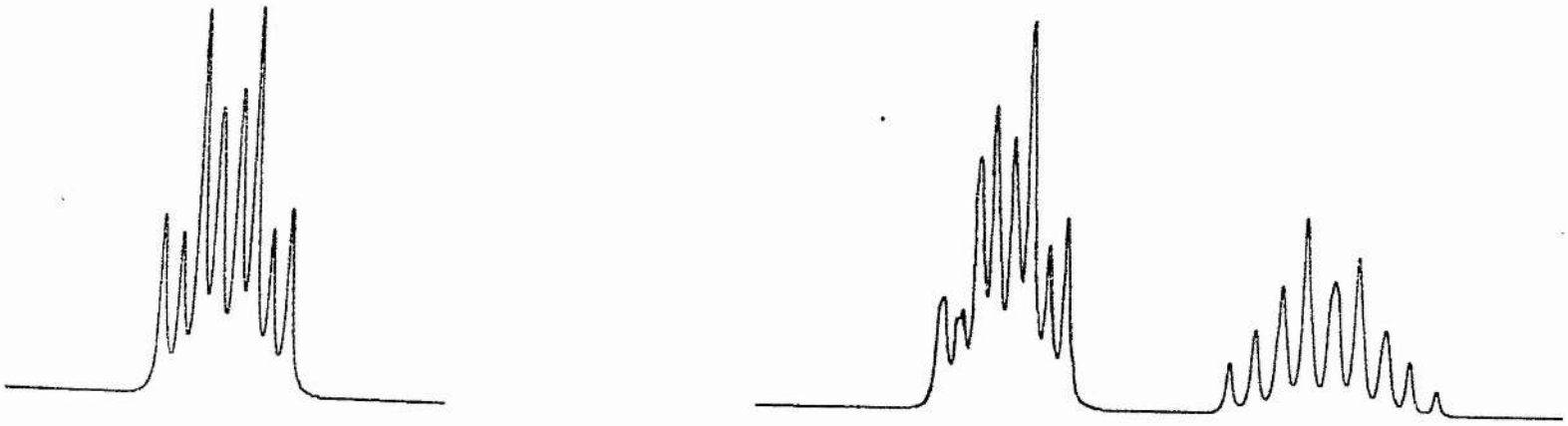


Figure 8 Combined spin simulation showing the correct admixture of AM_2X_2, AMX_2, and AM_2X spectra

3.3.3 REACTION OF ^{13}C-LABELLED HEXACYANOFERRATE(II) WITH AQUOCOBALAMIN; STUDIED BY ^{13}C HIGH FIELD NMR

To determine whether the binding of nitroprusside to cobalamin is specific to the nitrosyl ligand a series of similar experiments using hexacyanoferrate(II) instead of nitroprusside was undertaken.

In isotonic buffer the ^{13}C NMR spectrum of $[Fe(^{13}CN)_6]^{4-}$ consists of a singlet, having δ = 177.7ppm and $\nu_{1/2} < 5$Hz. Although the 90% enriched hexacyanoferrates-^{13}C is 35.4% $[Fe(^{12}CN)(^{13}CN)_5]^{4-}$ no resonance for this species was detected. Consequently, it can be concluded that the isotopic chemical shift difference between $[Fe(^{12}CN)(^{13}CN)_5]^{4-}$ and $[Fe(^{13}CN)_6]^{4-}$ is less than 0.05ppm. In addition the ^{13}C nuclei *cis* and *trans* to the ^{12}CN ligand of the former species are effectively isochronous at 90MHz ^{13}C resonance.

Initially, an equimolar quantity of H_2OCb (5.4×10^{-3} mol dm^{-3}) was added to hexacyanoferrate(II)-^{13}C and the resulting spectrum contained two singlets of approximately equal intensity (see Figure 9) assignable to free (δ = 177.7ppm) and bound (δ = 174.1ppm, $\nu_{1/2}$ = 20Hz) $[Fe(^{13}CN)_6]^{4-}$. When two molar equivalents of aquocobalamin were added to hexacyanoferrate(II) the singlet due to free $[Fe(^{13}CN)_6]^{4-}$ was almost completely suppressed, the bound $[Fe(^{13}CN)_6]^{4-}$ resonance at 174.1ppm remained and two new resonances appeared at 169.2ppm and 181.5ppm (Figure 10). When the molar ratio was increased to 1:5 $[Fe(^{13}CN)_6]^{4-}$ to aquocobalamin only the resonances at 169.2 and 181.5ppm remained (Figure 11). Both resonances were rather broad,

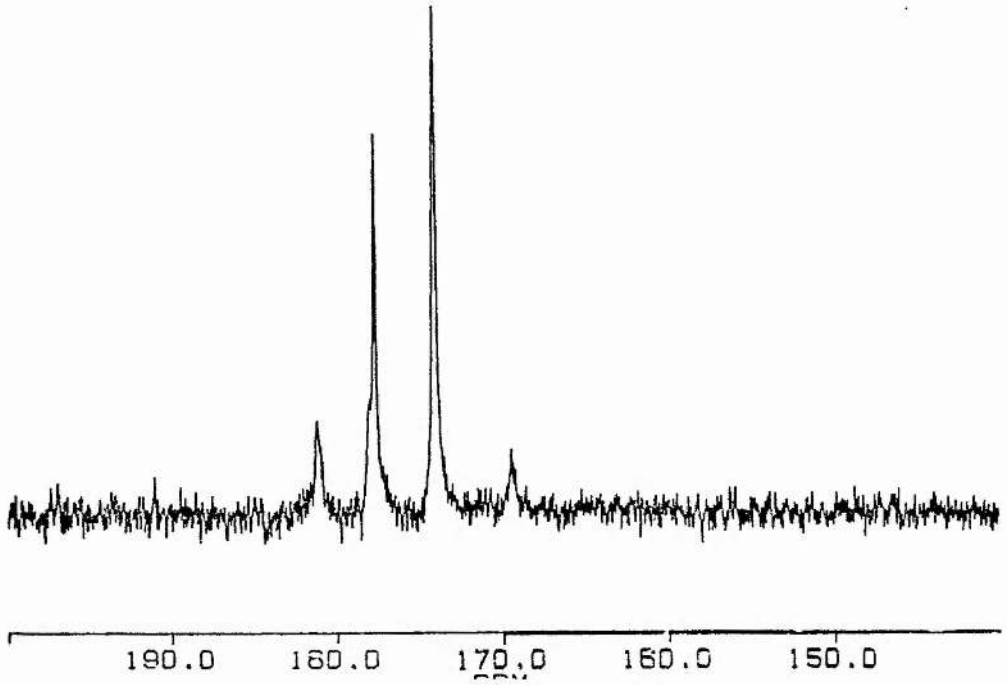


Figure 9 90MHz ^{13}C NMR spectrum of a 1:1 molar ratio of H_2OCb and 90% labelled sodium hexacyanoferrate(II) in isotonic buffer pH 7.2.

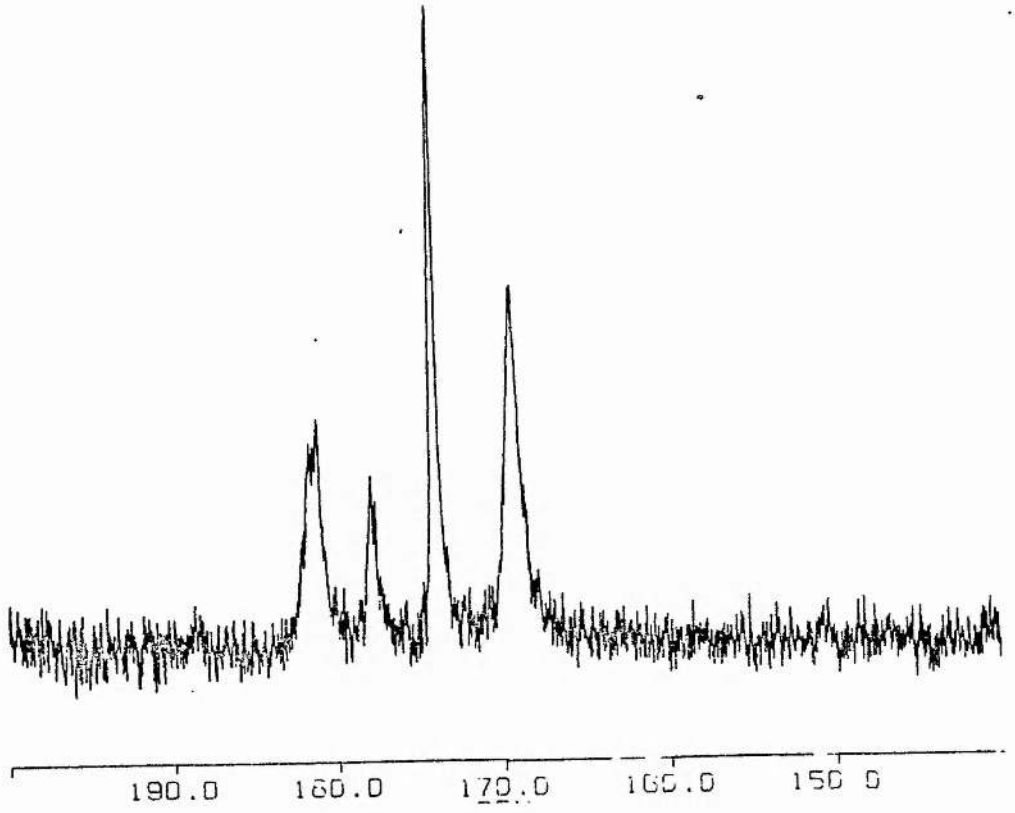


Figure 10 90MHz ^{13}C NMR spectrum of a 2:1 molar ratio of H_2OCb and 90% labelled sodium hexacyanoferrate(II) in isotonic buffer pH 7.2.

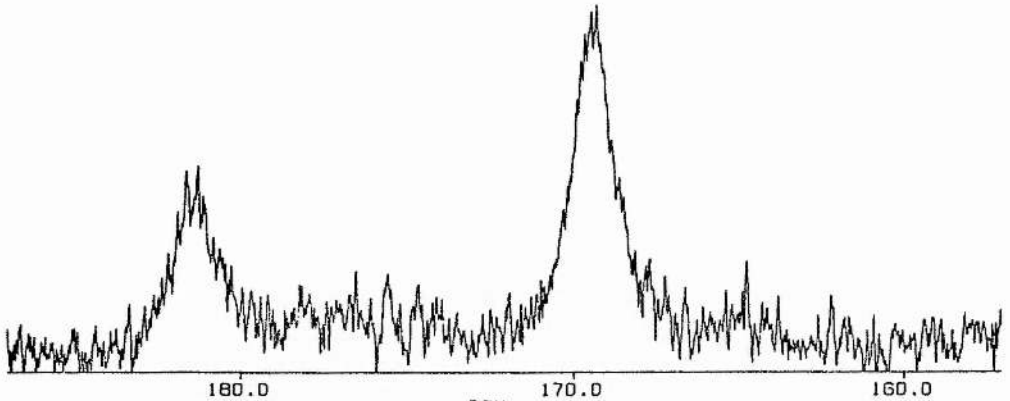


Figure 11 90MHz ^{13}C NMR spectrum of a 5:1 molar ratio of H_2OCb and 90% labelled sodium hexacyanoferrate(II) in isotonic buffer pH 7.2.

unresolved multiplets from which no coupling data could be obtained in the solution state. At five-fold molar excess of aquocobalamin the two resonances at 162.2 and 181.5ppm remained in the approximate intensity ratio of 2:1. As expected, when CNCb was used in place of H_2OCb the spectrum of $[Fe(^{13}CN)_6]^{4-}$ remained unperturbed at any molar ratio.

Although the solution state spectra of the $[Fe(CN)_6]^{4-}$:H_2OCb system do not allow definitive interpretation, it is evident that binding did occur, and it was prevented by blocking the axial site of the cobalt with cyanide (cyanocobalamin). The absence of resolvable fine structure did not allow positive identification of a 1:1 complex of hexacyanoferrate and aquocobalamin (Photograph 6) or of a 1:2 complex (Photograph 7). It is probable that the new complexes contain Fe-C-N-Co fragments and if the tentative assignments of the two new species as 1:1 and 1:2 complexes are correct, then the ^{13}C chemical shifts of the cyano ligands exhibited the same trends upon complexation as those in the nitroprusside (see Table 3).

In the case of free nitroprusside and its 1:1 (Species 1) and 1:2 (Species 2) adducts with cobalamin there was no fast exchange on the NMR time scale between the species. Upon change of the molar ratio of nitroprusside:H_2OCb the interconversion between these species occurred readily within, at most, a few minutes. The lack of resolvable fine structure in the spectra of $[Fe(^{13}CN)_6]^{4-}$ and it's complexes with cobalamin could be due to fluxional processes being faster in comparison to the $[Fe(CN)_5NO]^{2-}$:H_2OCb system. In the 1:1 complex, fast site exchange involving mobility of the cobalamin fragment over

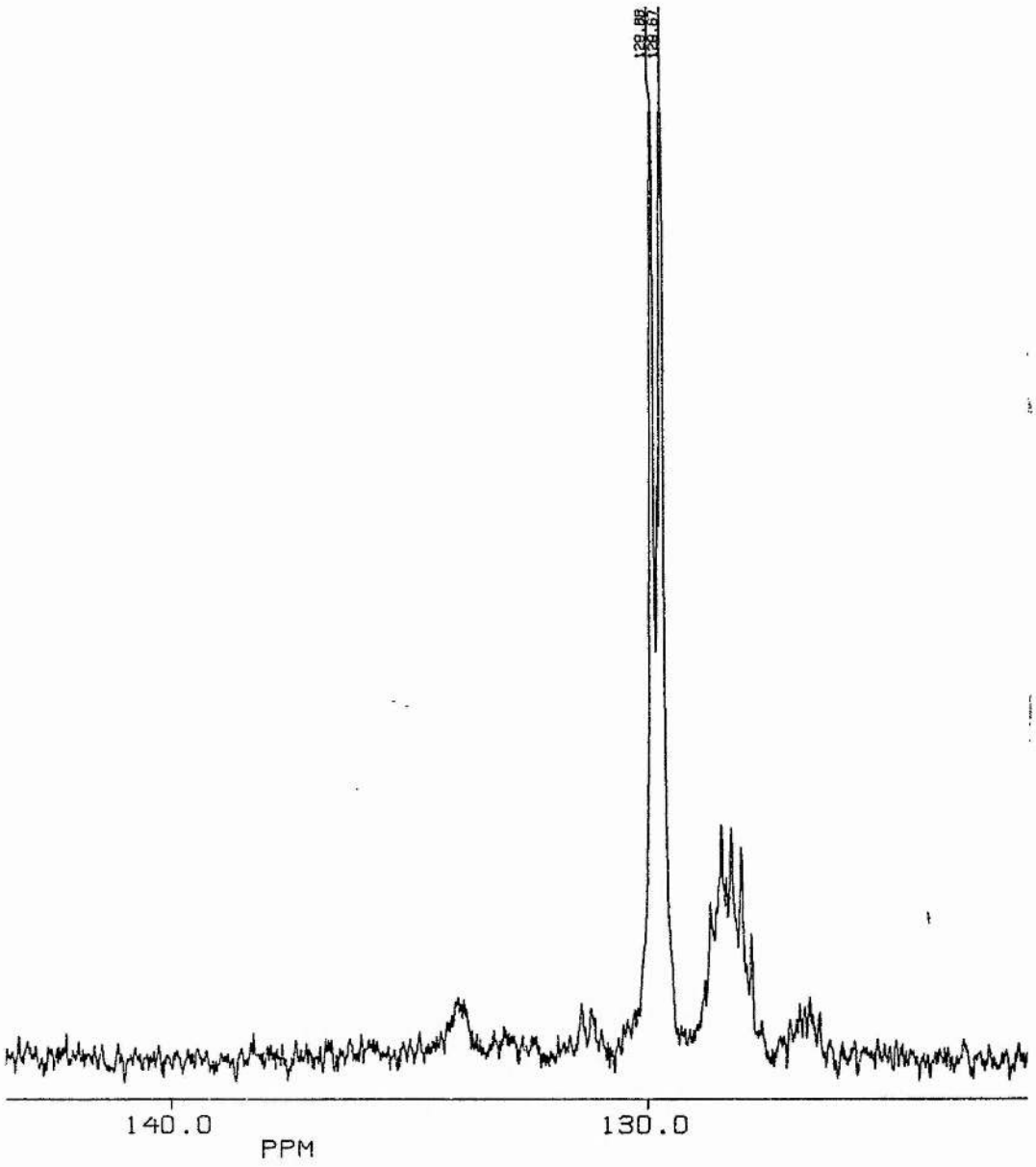


Figure 12 90MHz ^{13}C NMR spectrum of a 2:1 molar ratio of aquomethyl-cobaloxime and 90% labelled SNP in methanol-d_4.

six equivalent cyano ligands in $[Fe(CN)_6]^{4-}$ could account for the sharp singlet at 174.1ppm. Similarly, in the 1:2 complex the two broad unresolved signals at 169.2 and 181.5ppm could be due to the onset of site exchange within an A_2X_4 system. In conclusion the $[Fe(CN)_6]^{4-}$ spectra have proved that the nitrosyl ligand in the nitroprusside ion is not crucial to the binding to cobalamin. The occurrence of fluxionality in the $[Fe(CN)_6]^{4-}$:aquo cobalamin systems has not been adequately proven by these solution state ^{13}C NMR studies.

3.3.4 BINDING OF CYANOFERRATES TO OTHER COBALT COMPLEXES

Reaction of SNP with Aquomethylcobaloxime

In the chemical literature, the binding of nitroprusside to cobalamin via a bridging cyano ligand, i.e. Co-N-C-Fe, is similar to the intermediate isolated[36] from the redox reaction between $[Fe(III)(CN)_6]^{3-}$ and $[Co(II)(CN)_5]^{3-}$ of composition $[(CN)_5Co(III)-NCFe(II)(CN)_5]^{6-}$. Subsequently, a similar intermediate $[(EDTA)-Co(III)-NCFe(II)(CN)_5]^{5-}$ was detected[37] in the redox reaction of $[Fe(III)(CN)_6]^{3-}$ and $[Co(II)(EDTA)]^{2-}$. From both the crystal field model and the angular overlap model for an approximately square pyramidal fragment $Co(III)L_5$, containing spin-paired cobalt(III), both methods indicate that the LUMO is composed primarily of the d_{z^2} orbital of cobalt, directed along the four-fold symmetry axes. Consequently, nucleophilic anions, such as cyanoferrates, may be expected

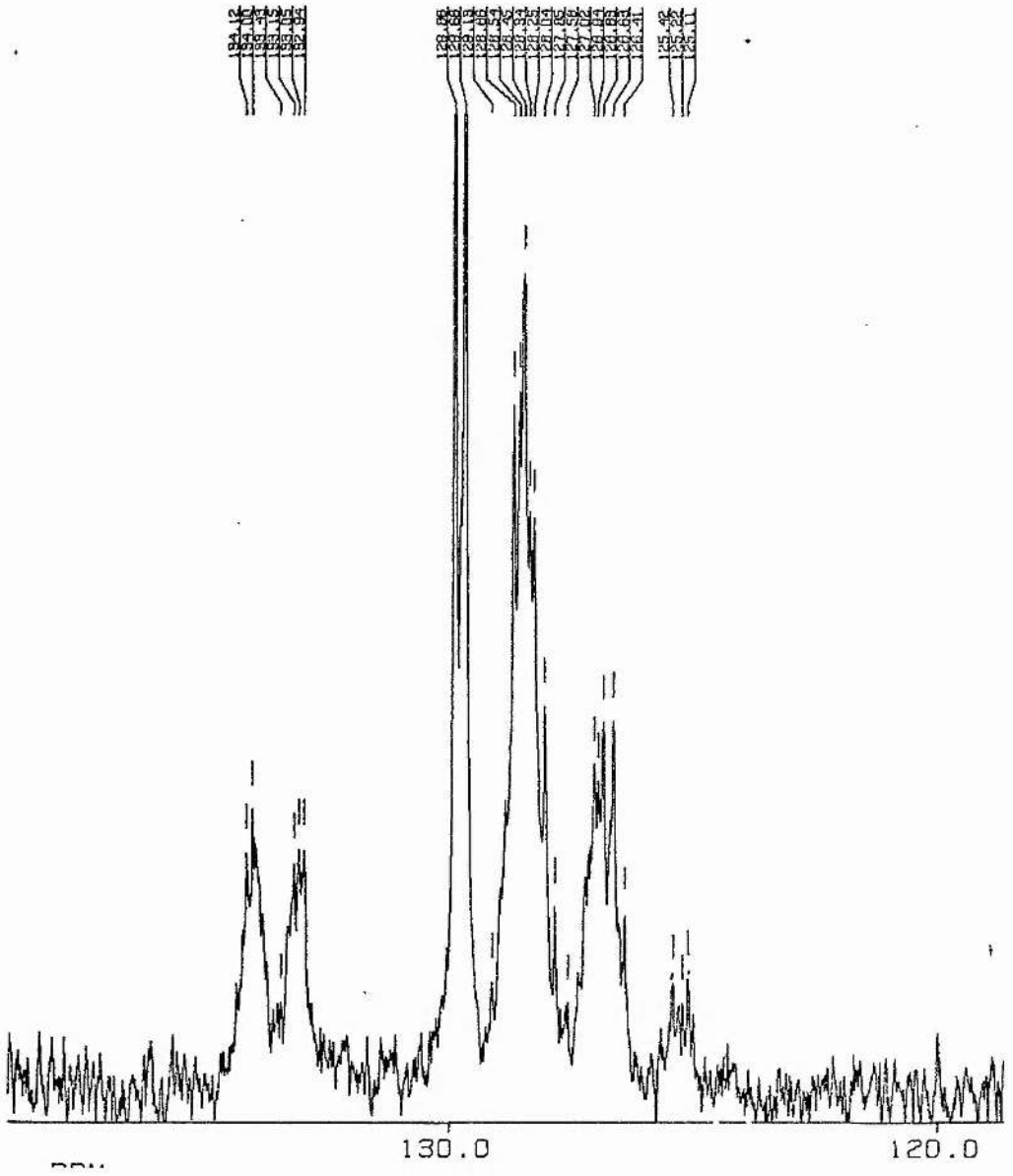


Figure 13 90MHz ^{13}C NMR spectrum of a 5:1 molar ratio of aquomethyl-cobaloxime and 90% labelled SNP in methanol-d_4.

to bind to other spin-paired cobalt(III) systems containing a readily displaceable water ligand. A series of ^{13}C NMR spectral studies was carried out using a variety of stoichiometric mixtures of either, $[Fe(CN)_5NO]^{2-}$ or $[Fe(CN)_6]^{4-}$, with an analogue of Vitamin B_{12a}, namely aquomethylcobaloxime $[CH_3Co(dmg)_2H_2O]$ (Photographs 8 and 9). In studying the spectral perturbation of nitroprusside caused by the addition of cobaloxime methanol-d_4 was used as the solvent since the solubility of the cobaloxime in phosphate buffer is very low. Initially, a spectrum of $[Fe(^{13}CN)_5NO]^{2-}$ in methanol-d_4 was recorded. This exhibited an AX_4 type spectrum characterised by δ_A= 128.2, δ_X= 129.7ppm, and J_{AX}= 18.3Hz. Incremental addition of aliquots of $[CH_3Co(dmg)_2H_2O]$ caused initially the appearance of a second AX_4 spectrum δ_A= 126.6, δ_X= 128.5ppm, and J_{AX}= 19.2Hz (see Figure 12). Only at higher ratios, $\geq$ 5:1 $[CH_3Co(dmg)_2H_2O]$:SNP, did a weak AM_2X_2 type spectrum appear (see Figure 13). Nitroprusside in the unbound form at such high ratios of Co:Fe is the major component of the ^{13}C NMR spectrum. It can be concluded that the binding between nitroprusside and cobaloxime is much weaker than in the nitroprusside:cobalamin system.

As in the nitroprusside:aquocobalamin interactions, the new AX_4 spectrum of the $[Fe(CN)_5NO]^{2-}$:$[CH_3Co(dmg)_2H_2O]$ was assigned to a 1:1 adduct with a Fe-C-N-Co moiety (Photograph 10). Chemical shift changes due to complexation of nitroprusside and aquomethylcobaloxime in the ^{13}C spectrum are small in comparison to the aquocobalamin complexes. Nevertheless for comparison the ^{59}Co chemical shift of $[CH_3Co(dmg)_2-H_2O]$ in methanol was recorded relative to $K_3[Co(CN)_6]$ and found to be 4270ppm (Figure 14a). On addition of two-fold molar

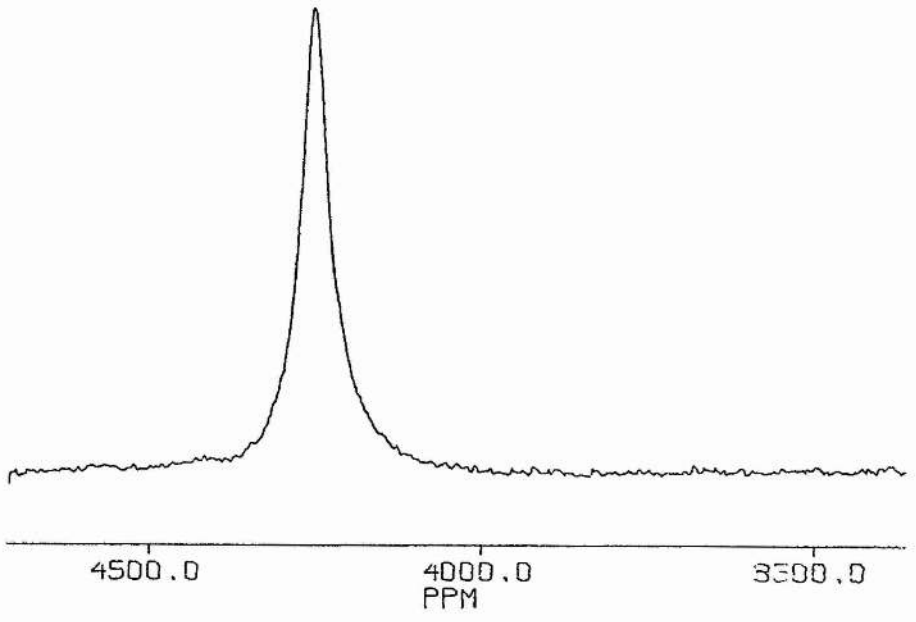


Figure 14a 85MHz ^{59}Co NMR spectrum of aquomethylcobaloxime in methanol-d_4.

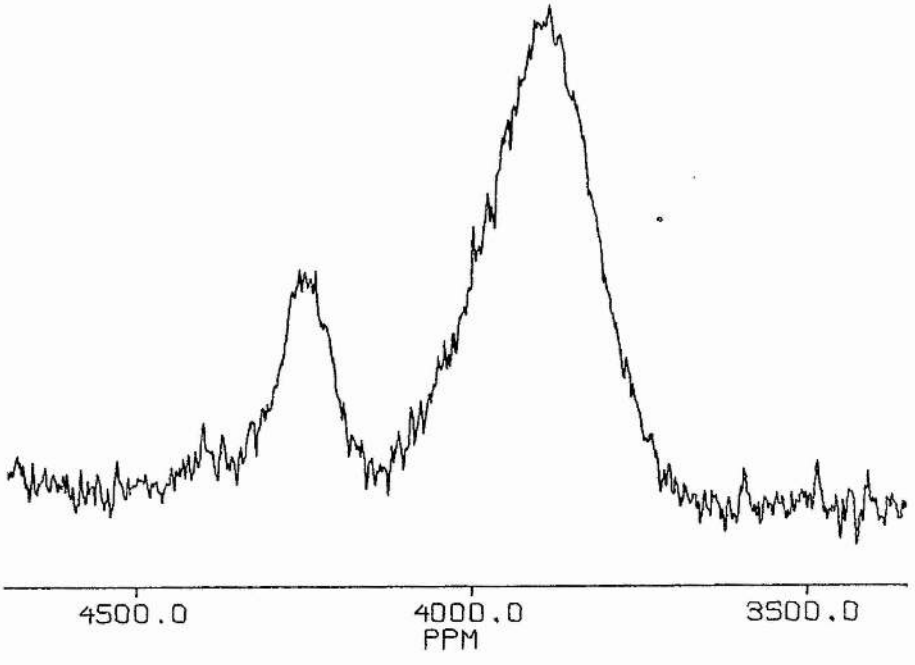


Figure 14b 85MHz ^{59}Co NMR spectrum of a 1:2 molar ratio of aquomethylcobaloxime and SNP in methanol-d_4.

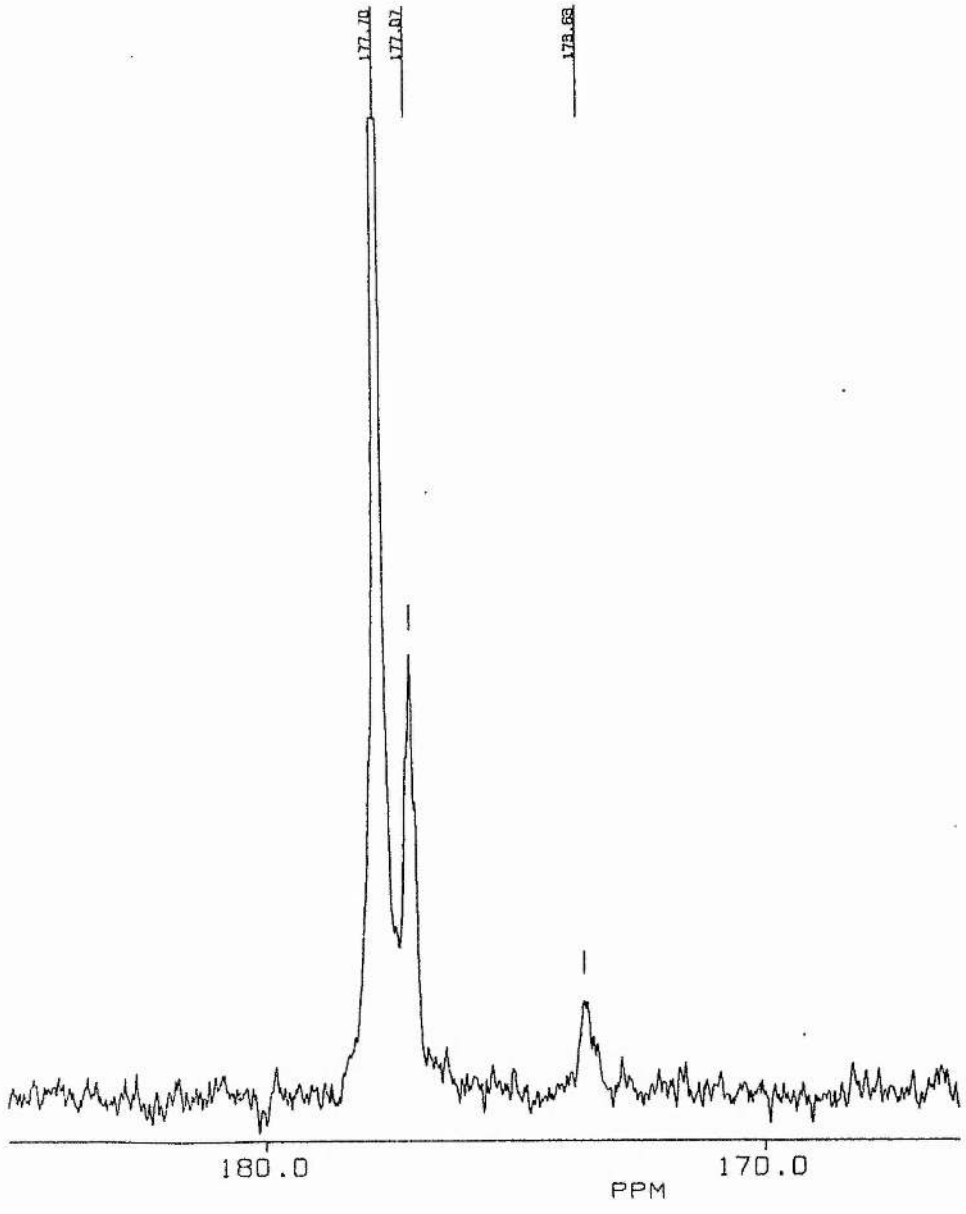


Figure 15 90MHz ^{13}C NMR spectrum of a 1:1 molar ratio of aquomethyl-cobaloxime and 90% labelled sodium hexacyanoferrate(II) in isotonic buffer pH 7.2.

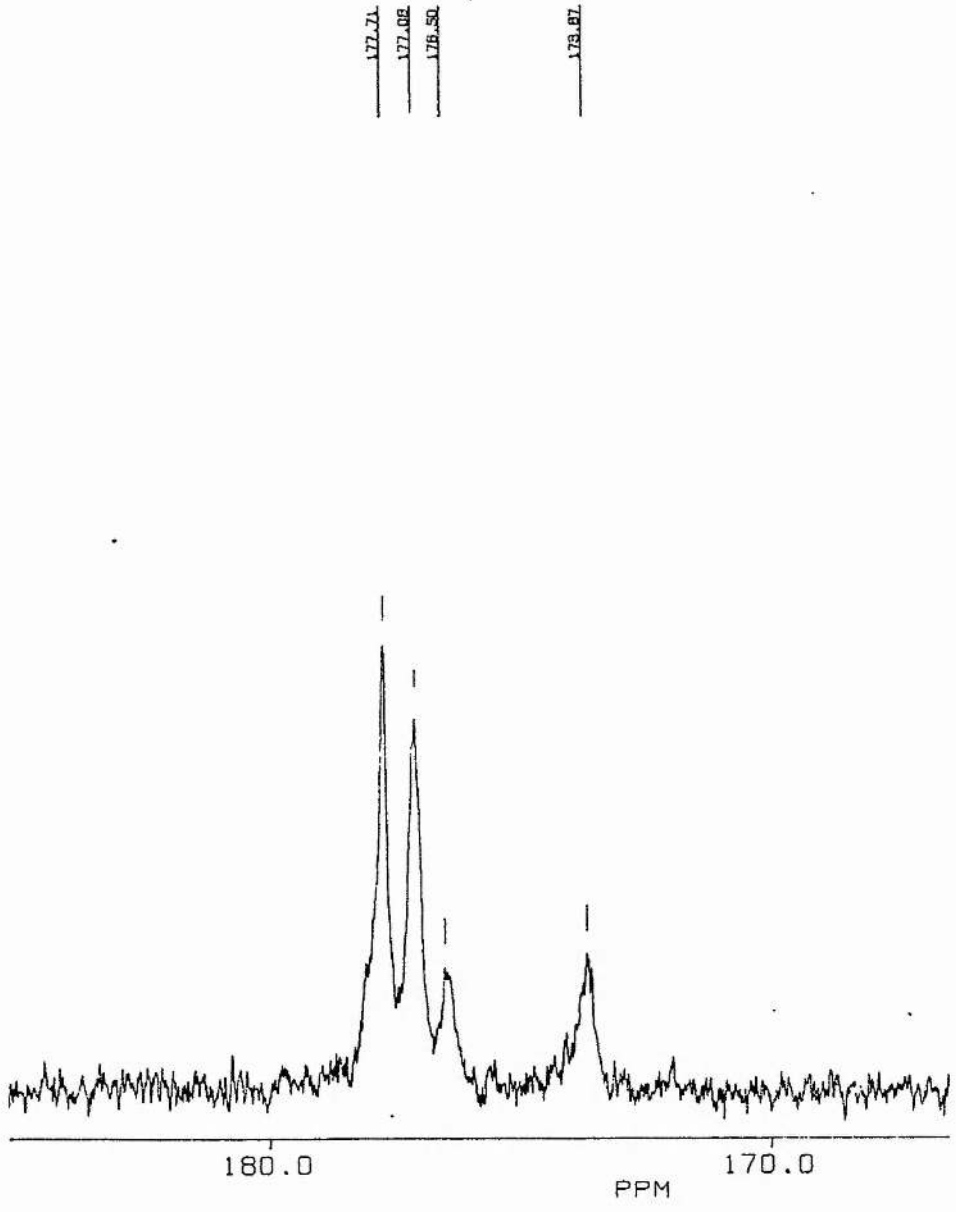


Figure 16 90MHz ^{13}C NMR spectrum of a 5:1 molar ratio of aquomethyl-cobaloxime and 90% labelled sodium hexacyanoferrate(II) in isotonic buffer pH 7.2.

excess of nitroprusside the resonance at 4270ppm was almost completely replaced by a new resonance at 3890ppm (Figure 14b). Therefore, the 1:1 adduct formation is real. Figure 13 exhibits the probable formation of an AM_2X_2 type system at high ($\geq$ 5:1) ratios of Co:Fe indicating a 2:1 complex (Photograph 11).

Interaction of Hexacyanoferrate(II) with $[CH_3Co(dmg)_2H_2O]$

To assess the necessity of the nitrosyl group of nitroprusside in the formation of $[CH_3Co(dmg)_2H_2O]$:$[Fe(CN)_5NO]^{2-}$ complexes, a series of ^{13}C NMR spectra were recorded of various molar ratios of $[Fe(CN)_6]^{4-}$ and aquomethylcobaloxime in phosphate buffer, pH 7.2. In the spectrum of a 1:1 mixture, a singlet δ = 177.7ppm assigned to unbound $[Fe(^{13}CN)_6]^{4-}$ was the major component. At less than one-tenth the intensity of the singlet at 177.7ppm a second singlet δ = 177.1ppm was present (Figure 15). Two new weak resonances appeared at δ = 173.9 and δ = 178.5ppm in an approximate 2:1 intensity ratio respectively at high ratios ($>$ 5:1) of Co:Fe (Figure 16). Although the lack of resolvable couplings prevents definite interpretation, the new resonances may indicate 1:1 (Photographs 12 and 13) and 1:2 (Photographs 14 and 15) hexacyanoferrate:aquomethylcobaloxime complexes. Therefore the nitrosyl group is not crucial for the complexing of cyanoferrates with the Vitamin B_{12a} analogue $[CH_3Co(dmg)_2H_2O]$ (see Table 3).

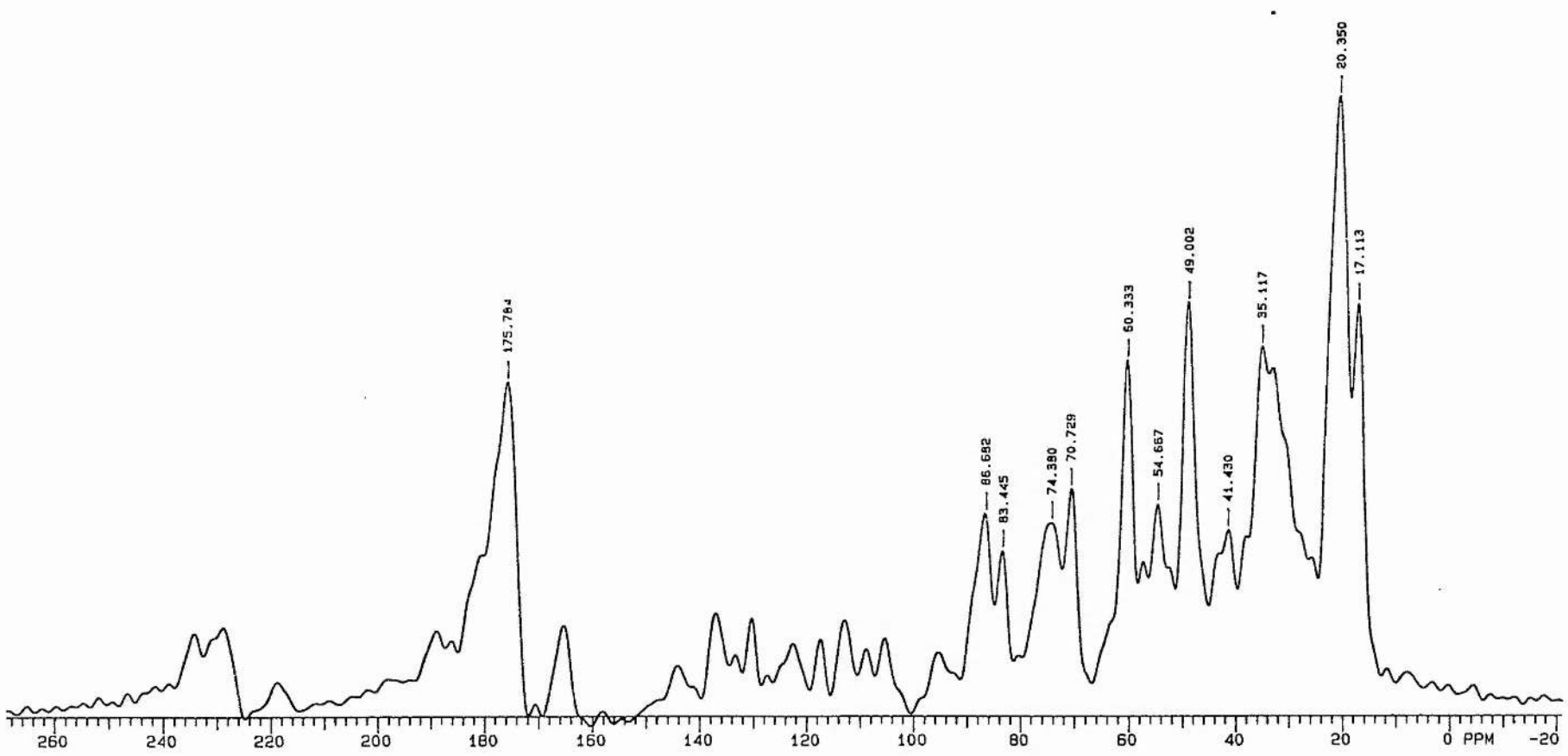


Figure 17 75MHz ^{13}C solid state CPMAS NMR spectrum of aquocobalamin.

3.3.5 SOLID-STATE ^{13}C NMR

As a result of the solution state ^{13}C NMR obtained showing a possible fluxional interaction of ^{13}C-labelled sodium hexacyanoferrate and H_2OCb (see p. 59) a solid state NMR study was undertaken to investigate the interaction of a 1:1 mole ratio mixture of hexacyanoferrate and H_2OCb.

Initially a Cross Polarisation Magic Angle Spinning (CPMAS)[8] ^{13}C solid state NMR spectrum was obtained of Vitamin B_{12a}, aquocobalamin (Figure 17). Chemical shifts were assigned (see Table 4 and Figure 18) by comparison with the solution state spectrum of cyanocobalamin.[1b,18,39] A solid state CPMAS spectrum recorded of $Na_4[Fe(II)(^{13}CN)_6].10H_2O$ is shown in Figure 19. In this mode the spectrum is complicated with a large number of spinning side bands (designated *). These spinning side bands are present in such large numbers because the sodium hexacyanoferrate is 90% ^{13}C-labelled. In the hexacyanoferrate spectrum only those peaks at 187.9, 174.5, and 171ppm (relative to TMS) are centre bands. Integration of the side bands and centre peaks gives a ratio of 0.57 for the resonance at 187.9ppm to the combined integration of 1.00 for the peaks at 174.5 and 171ppm. These three peaks may correspond to the three unique cyanide environments of the crystal structure of sodium hexacyanoferrate.[40] In the crystal structure there are three crystallographically different cyanide groups, i.e. three pairs of mutually *trans* cyano ligands. These distinct groups show minor variations in the Fe-C-N dimensions, but more importantly they differ

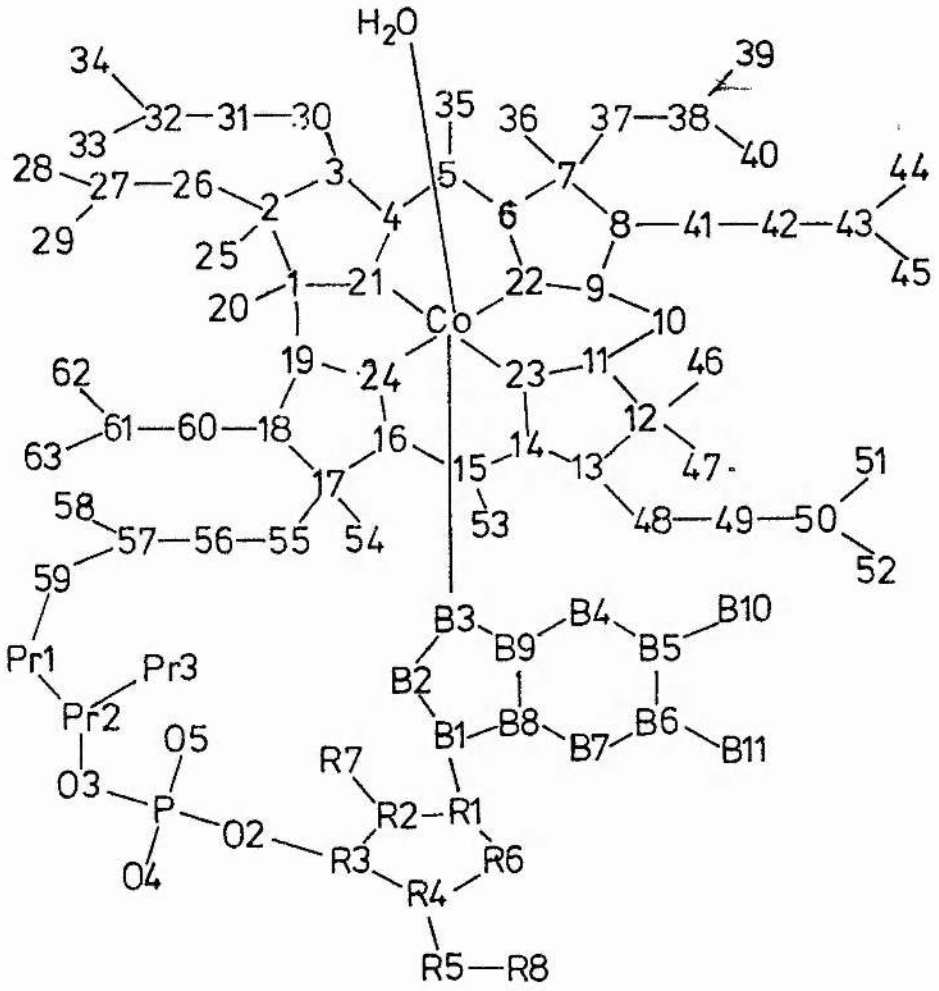


Figure 18 The IUPAC numbering scheme illustrating the atomic connectivity of aquocobalamin (hydrogen atoms omitted).

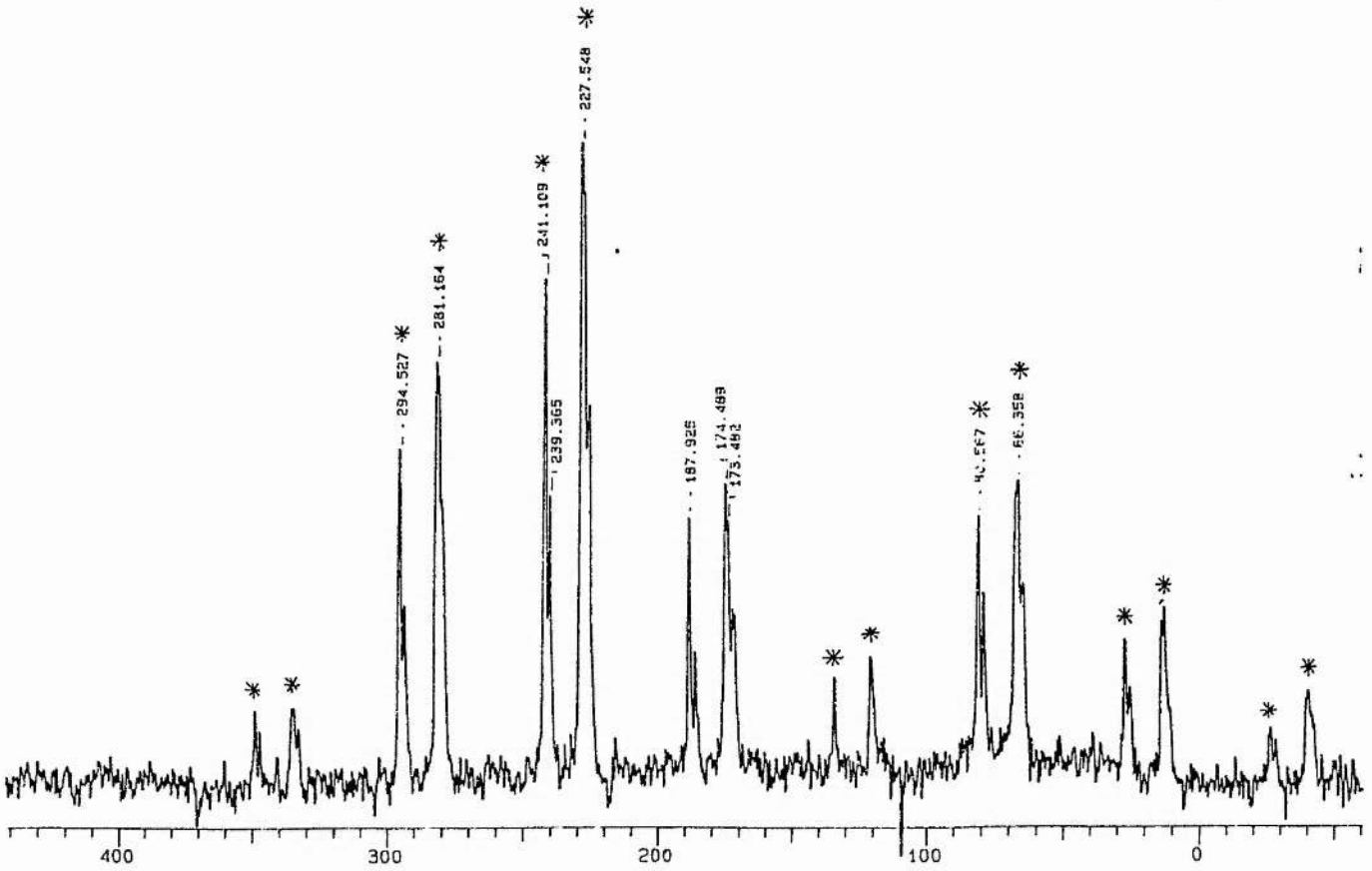


Figure 19 75MHz ^{13}C solid state CPMAS NMR spectrum of 90% labelled sodium hexacyanoferrate(II) (* designates spinning side bands).

in the co-ordination of each to the terminal nitrogen atoms. In each $[Fe(II)(^{13}CN)_6]^{4-}$ octahedron in one pair of cyano ligands the two nitrogens are each co-ordinated to three sodium ions, the nitrogen atoms of two other cyano ligands are each co-ordinated to one sodium ion and form a hydrogen bond with one molecule of water, the terminal nitrogen atoms of the third pair of cyano ligands form hydrogen bonds to three water molecules.

Table 4

Assignments refer to numbering in Figure 18.

Solution State (ppm)	Solid State (ppm)	Assignment
	234.5	32, 38, 43
	229	50, 27, 57
	218.5	
179.9, 180.9	181	4, 16
174.4	175.78	9
166.2	165.5	14
142.7	144.0	B2
137.6	137.0	B9
135.9	133.5	B8
133.8, 130.8	130.0	B5, B6
117.4	117.5	B4
112.3	112.5	B7
108.4	109.0	5
105.0	105.0	15
95.8	96.0	10
86.2, 88.1	86.68	1, R1
83.1	83.44	R4
74.3, 74.0	74.38	R2, R3
70.0	70.73	Pr2
60.2	60.33	2
57.6	56.50	3
56.8, 55.0	54.67	8, 13
52.3	52.0	7
49.2, 48.4	49.0	12, 17
46.5	44.0	Pr1
43.7, 43.9	43.5	Acetamide CH_2
40.0	41.43	18
35.8, 35.5	35.11	Propionamide, Acetamide CH_2
31.4, 31.1, 32.2, 32.6	32.0	47, 55, Acetamide CH_2
29.9	29	48
26.8	26.5	30, 41
19.9	20.35	46, 20, 25
16.3, 17.7, 16.7	17.11	35, 36, 54

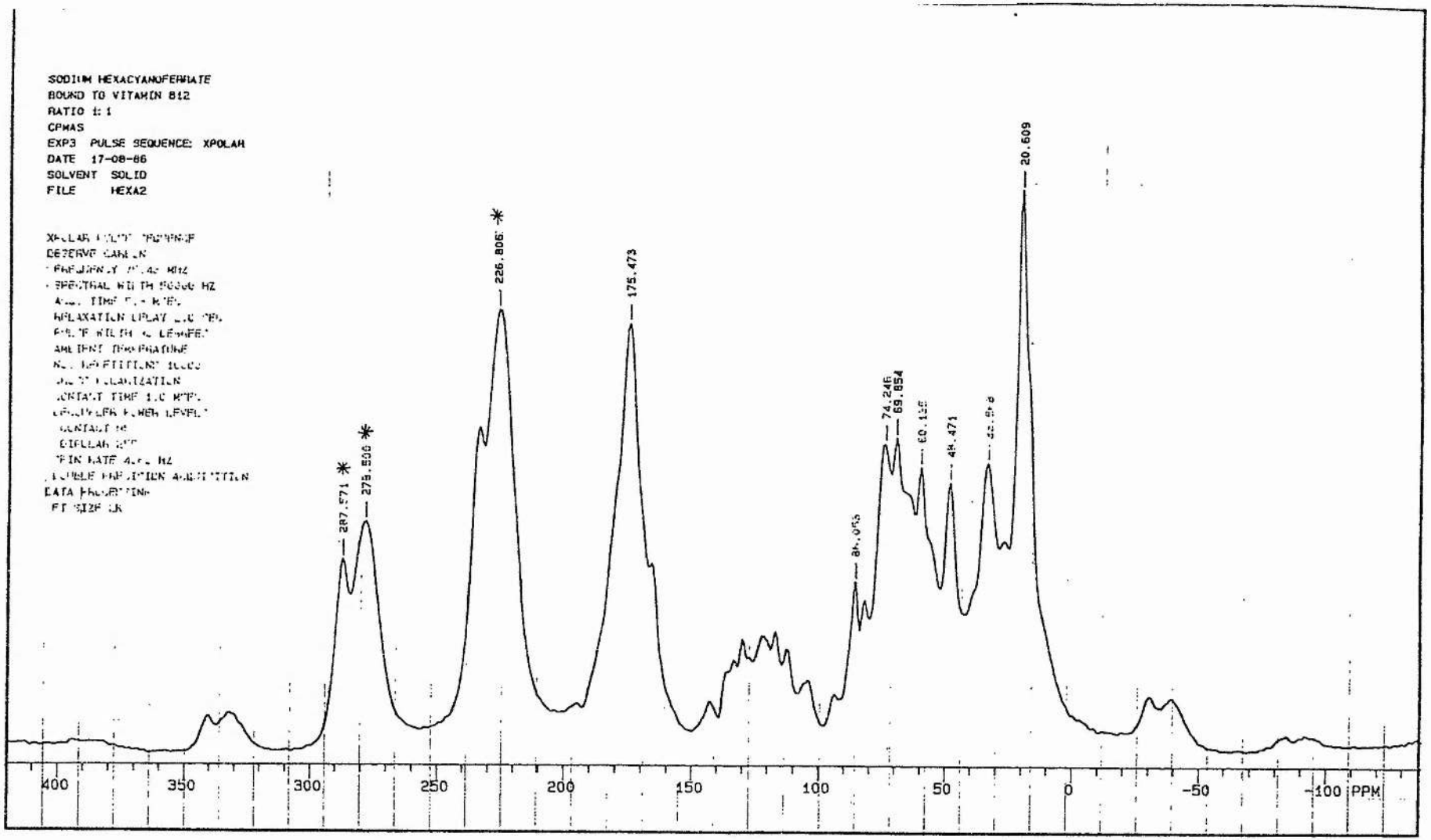


Figure 20 75MHz ^{13}C solid state CPMAS NMR spectrum of a 1:1 molar ratio of H_2OCb and 90% labelled sodium hexacyanoferrate(II).

The solution state ^{13}C NMR spectrum of sodium hexacyanoferrate consists of a singlet, δ = 177.7ppm and $\nu_{1/2}$= 5Hz. This singlet is an average of the three independent singlets noted in the ^{13}C solid state NMR spectrum.

When a ^{13}C solid state NMR spectrum of 1:1 mole ratio of ^{13}C-labelled hexacyanoferrate was recorded using CPMAS the peaks at 287.57, 278.5, 266.8, and 175.47ppm became very broad (see Figure 20) indicating a decrease in resolution in comparison with the indiviual CPMAS spectra of both hexacyanoferrate and aquocobalamin. This decrease in resolution indicates a decrease of crystallinity and hence binding interactions of the two species. To simplify the spectrum of the sample, a solid state ^{13}C NMR spectrum was recorded using both CPMAS and TOSS modes[8] (Figure 21a). As a result the three broad peaks at 287.57, 278.5, and 266.8ppm of the CPMAS spectrum (Figure 20) disappeared, leaving a broad resonance at 178.1ppm. This peak corresponds to the centre band of the ^{13}C-labelled hexacyanoferrate (see Figure 19) and C_{16}, C_4, and C_9 of the cobalamin, i.e. the carbons in the corrin ring directly attached to a nitrogen atom by a double bond (see Table 4 and Figures 17 and 18). In an attempt to further simplify the spectrum the sample was subjected to CPMAS and NQS[8] modes (see Figure 21b). This removes peaks from the spectrum which correspond to non-quaternary carbon atoms, with the exception of the methyl carbons. This resulted in the loss of the propionamide β-CH_2, the acetamide CH_2, C_{18}, C_8, C_{13}, etc of the corrin ring, but the resonance at 175.4ppm remained along with the three spinning side bands.

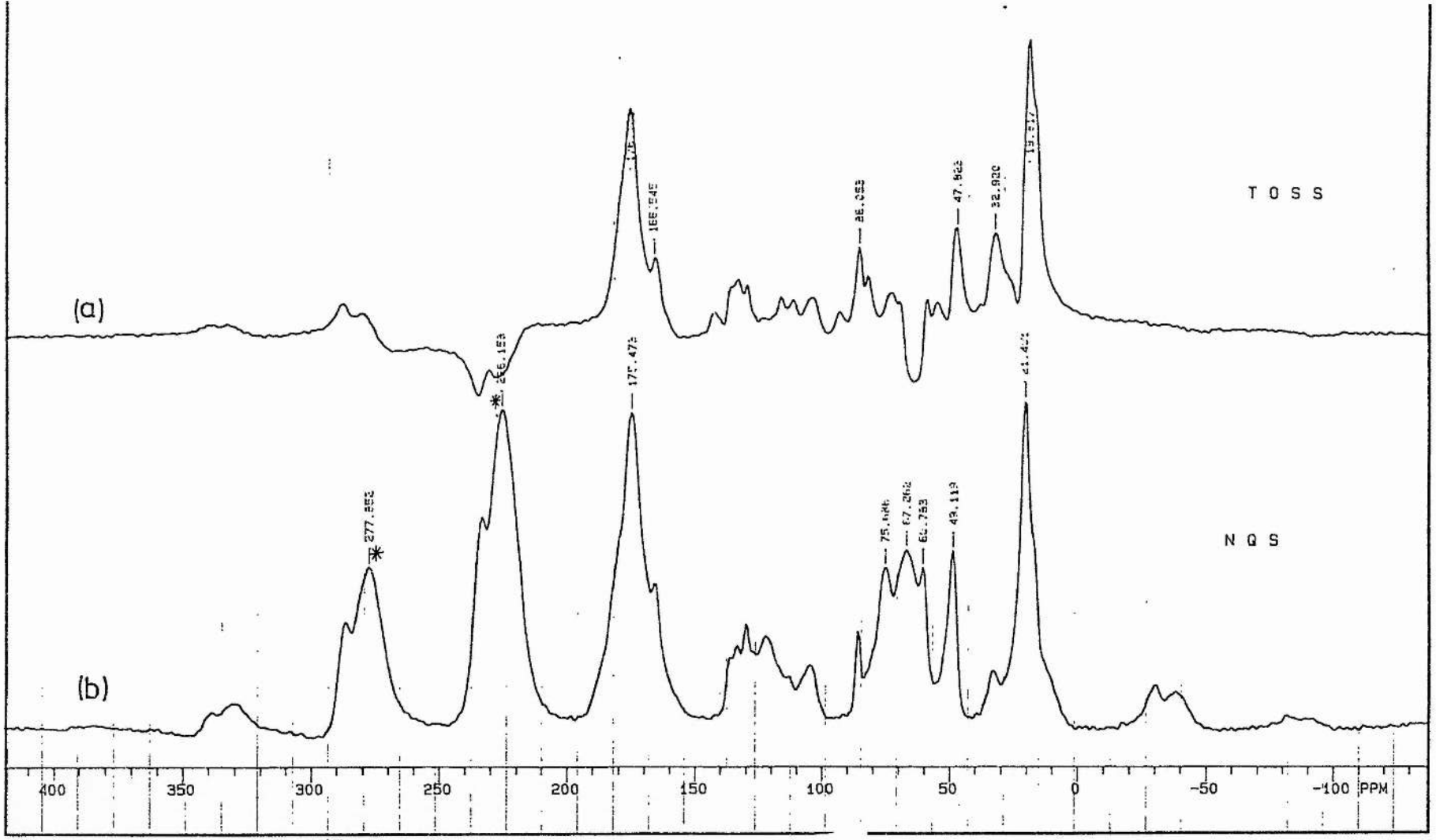


Figure 21a and 21b 75MHz ^{13}C solid state CPMAS NMR spectrum of a 1:1 molar ratio of H_2OCb and 90% labelled sodium hexacyanoferrate(II) run in TOSS mode (21a) and NQS mode (21b).

In comparing all the spectra of the 1:1 mole ratio hexacyanoferrate: H_2OCb (Figures 20 and 21a,b) it is apparent that the carbon atoms attached directly by double bonds to the ligating nitrogens of the corrin ring are always going to have a similar resonance to the ^{13}CN of the hexacyanoferrate making interpretation difficult. Nevertheless, the fact that the spinning side bands and the centre band of the hexacyanoferrate have become very broad in comparison with the line widths of the carbons of unbound hexacyanoferrate and cobalamin observed between indicates not only binding, but that at 25°C there is still fluxional behaviour in the 1:1 complex on the NMR timescale in the solid state. There is further evidence of interaction found by comparing the CPMAS spectrum of H_2OCb (Figure 17) with the 1:1 sample CPMAS spectrum (Figure 20). There are slight changes in chemical shift and general shape of the peaks in the region of 20 to 87ppm in the 1:1 sample of hexacyanoferrate:H_2OCb. This region includes the peripheral acetamide and propionamide carbons as well as several carbons of the corrin ring. The presence of 90% ^{13}C labelled hexacyanoferrate in the 1:1 sample has enhanced the carbon atom resonances of the cobalamin, this is due to a polarisation effect between the two moieties.

In conclusion, both in solution and solid state, there is evidence of binding in a 1:1 mole ratio of sodium hexacyanoferrate and aquocobalamin. In both solution and solid state the 1:1 complex exhibits fluxional behaviour, fast site exchange on the NMR timescale, involving mobility of the cobalamin fragment over the six equivalent ligands of the $[Fe(II)(^{13}CN)_6]^{4-}$ anion.

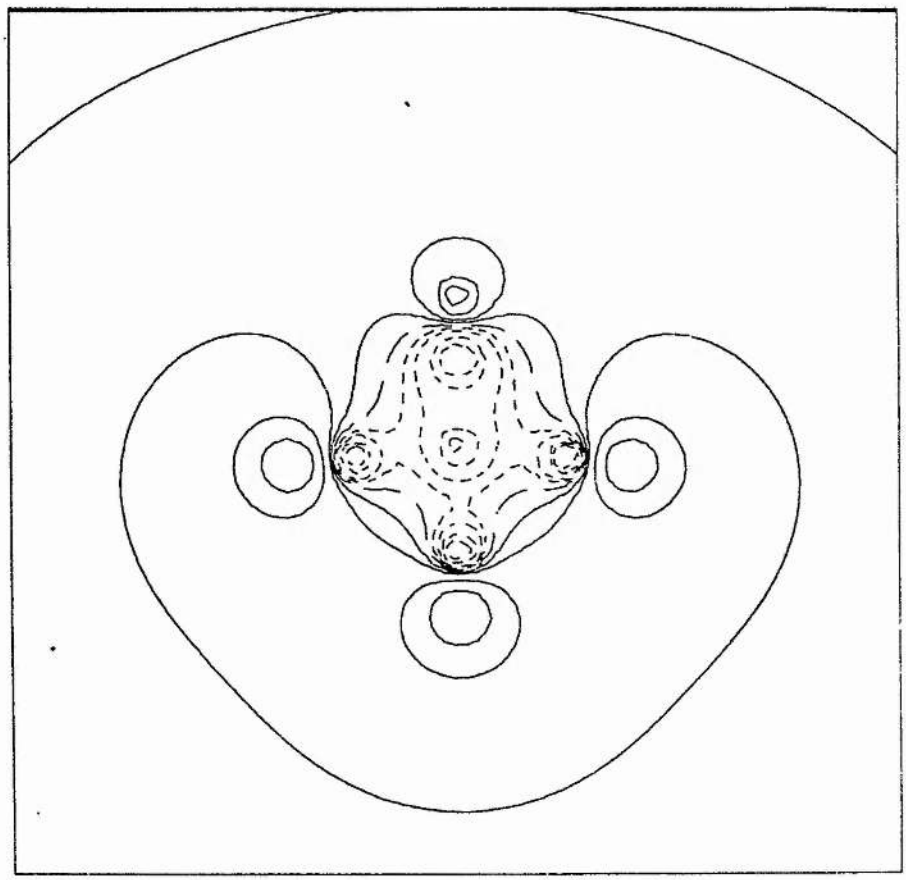


Figure 22 Electrostatic potential in the σ_v plane of $[Fe(CN)_5NO]^{2-}$. Successive contours represent -1.0, -0.5, -0.2, -0.1, 0.0, 0.1, 0.2, 0.5, and 1.0 a.u. (———— negative, — — — zero, - - - - - positive).

3.3.6 THE BINDING OF CYANOFERRATES TO COBALOXIMES AND COBALAMINS A STUDY USING EXTENDED HUCKEL MOLECULAR ORBITAL CALCULATIONS

As a result of the ^{13}C and ^{59}Co solution and solid state NMR studies certain questions required answering about these complex formations;

(i) the geometry of the Fe-CN-Co bridge,

(ii) the relative energies of the possible isomeric forms of the nitroprusside complexes,

(iii) the nature of the bonding in the complexes, especially the orbital interactions involved,

(iv) the greater stability of the cobalamin compared with the cobaloxime complexes.

Before the EHMO study can be applied to the complex formations, the nitroprusside ion, hexacyanoferrate(II), the corrin ring, and aquo-methylcobaloxime must be studied individually.

Nitroprusside and Hexacyanoferrate

In the case of the nitroprusside anion, EHMO calculations showed that the d_{xy} orbital is the HOMO at -12.79eV, followed next by the equatorial σ(FeCN) combination of type E in C_{4v} symmetry at -12.95eV: the equatorial σ(FeCN) levels of type A_1 and B_1 lie at -13.95 and 13.76eV respectively. The d_{yz} and d_{zx} orbitals are at -13.12eV. The energy levels which turn out to be most important in the binding to cobalt are the axial σ(FeCN) and π(CN) which are at -13.33 and -14.49eV respectively. In the nitroprusside anion,

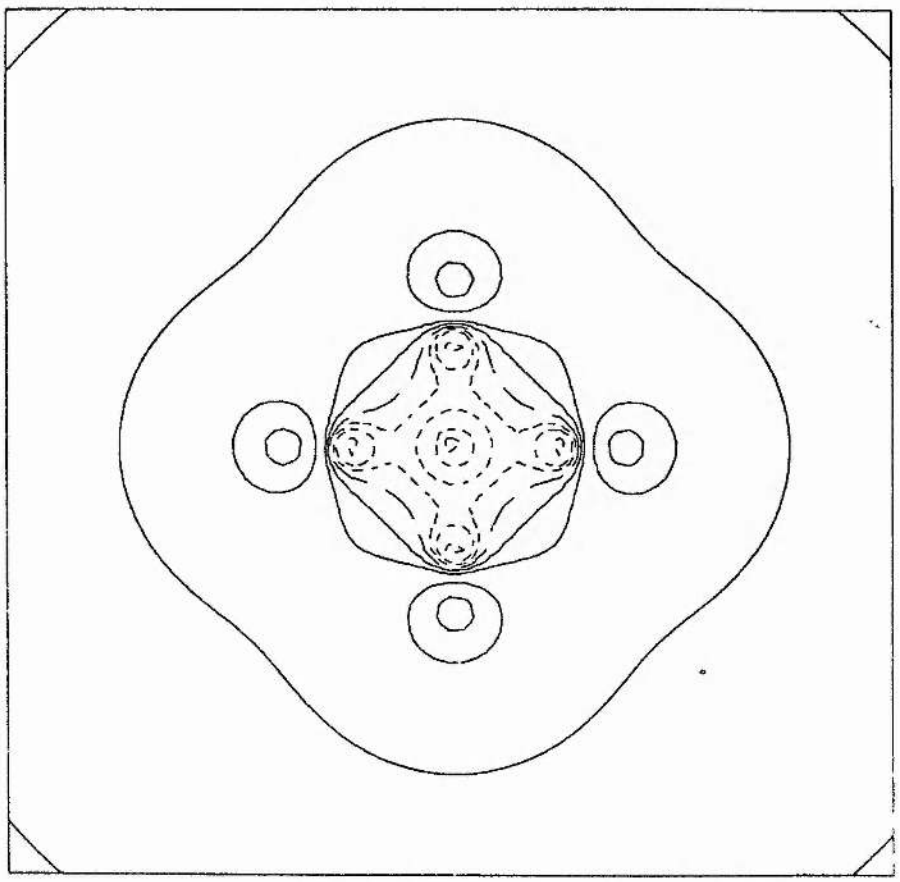


Figure 23 Electrostatic potential in the equatorial plane of $[Fe(CN)_5NO]^{2-}$. Successive contours represent -1.0, -0.5, -0.2, -0.1, 0.0, 0.1, 0.2, 0.5, and 1.0 a.u. (————negative, — — —zero, - - - - -positive).

$[Fe(CN)_5NO]^{2-}$, the π^*(NO) orbital at -10.01eV was calculated to be the LUMO. As expected for an anti-bonding orbital the coefficients indicate fairly strong localisation on the nitrogen atom. The axial π^*(CN) orbital at -8.45eV is the next above the LUMO.

When considering the relative charges of the ligands of the anion, the terminal oxygen of the nitrosyl ligand is calculated to carry a charge of -0.42e. The nitrogen atom of the nitrosyl ligand has the highest positive charge, +0.72e, of any atom in the nitroprusside anion (the equatorial carbons bear charges of +0.46e and the axial carbon a charge of +0.51e). The nitrogen atoms of the cyano ligands are calculated to be the most negatively charged; the axial cyano nitrogen carries a charge of -1.07e and the equatorial nitrogens slightly less at -0.98e. Therefore, it can be concluded that electrophilic reagents will interact primarily, if not exclusively, at the terminal atoms of the cyano ligands, with perhaps slight preference for the axial site over the equatorial. In contrast, nucleophilic reagents are expected to attack, whether in orbital-controlled or in charge-controlled reactions, exclusively at the nitrosyl nitrogen, as reported previously.[41-44]

A section in one of the σ_v planes, which includes the axial nitrosyl, axial cyanide and two of the equatorial cyanides is shown in Figure 22. An alternative section through the equatorial plane of the nitroprusside anion is shown in Figure 23.

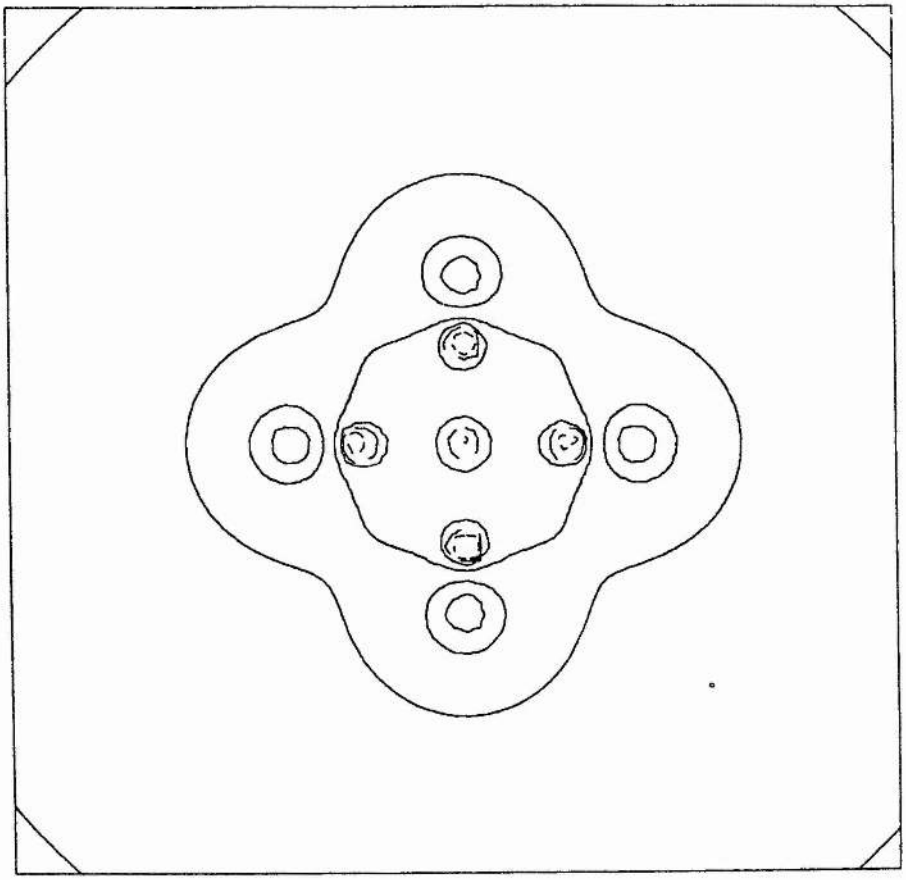


Figure 24 Electrostatic potential in the σ_h plane of $[Fe(CN)_6]^{4-}$. Successive contours represent -2.0, -1.0, -0.5, 0.0, and 0.5 a.u. (——— negative, — — — zero, - - - - - positive).

In the case of the hexacyanoferrate(II) anion of O_h symmetry, EHMO calculations showed the HOMO at -12.83eV is comprised of d_{xy}, d_{yz}, and d_{zx} orbitals of symmetry class T_{2g}. Directly below the HOMO lie T_{1u}, E_g, and A_{1g} combinations of the $\sigma(Fe(CN)_5$ orbitals at -13.04, -13.91, and -14.12eV respectively. There are four sets of $\pi^*(CN)$ orbitals of the symmetry classes T_{2u}, T_{1u}, T_{2g}, and T_{1g} at -8.32, -8.16, -7.89, and -7.54eV respectively positioned above the large HOMO-LUMO gap. In the hexacyanoferrate(II) anion the carbon atoms bear the charge of +0.44e and the nitrogens a charge of -1.15e. A section in one of the σ_h planes of $[Fe(CN)_6]^{4-}$ which includes the iron and four coplanar ligands is represented in Figure 24.

In comparing the sectional plots of electrostatic potentials around $[Fe(CN)_5NO]^{2-}$ (Figures 22 and 23) and $[Fe(CN)_6]^{4-}$ (Figure 24) anions the striking feature in Figure 22 is the very electrophilic reactive site at the nitrosyl ligand. The electrostatic potential sections illustrated in Figures 23 and 24 are relatively featureless in comparison. In every section, the nucleophilic sites of reaction for both these anions were seen to be the peripheral nitrogens of the cyanide ligands.

Aquomethylcobaloxime and Methylcobaloxime

The atomic co-ordinates for aquomethylcobaloxime, $CH_3Co(dmg)_2H_2O$, were taken from the X-ray analysis of McFadden and McPhail.[13] Regardless of the axial ligand identical co-ordinates were used throughout the calculations for the $CH_3Co(dmg)_2$ portion. As pre-

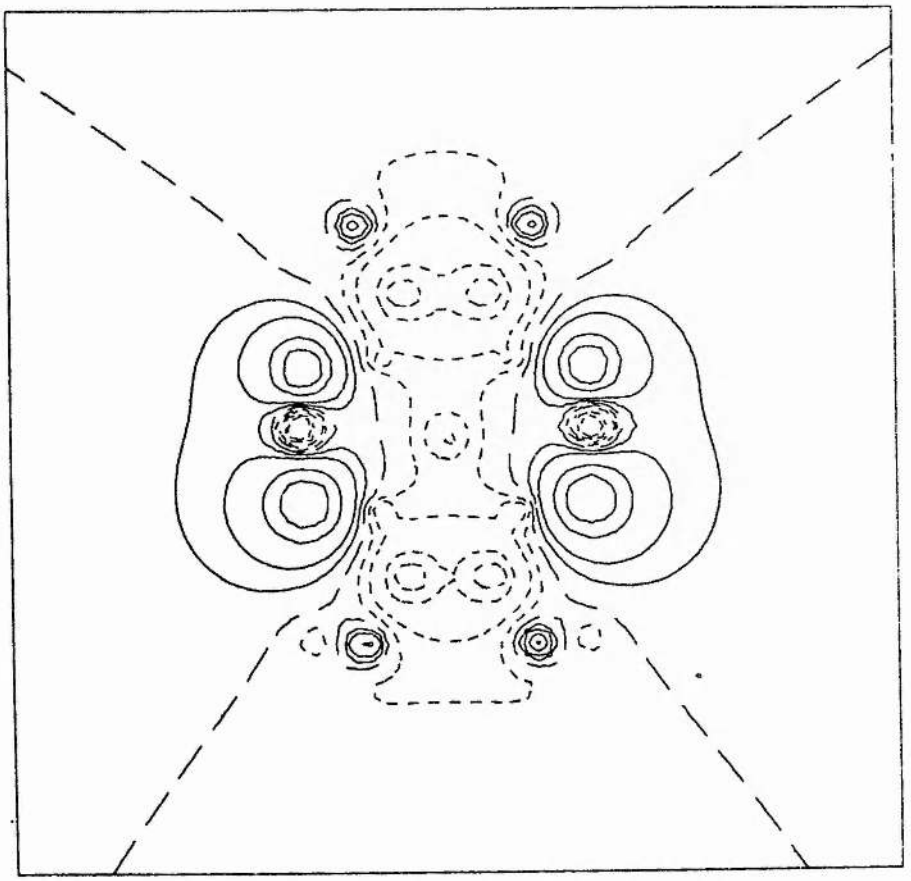


Figure 25 Electrostatic potential in the xy plane of $CH_3Co(dmg)_2$. Successive contours represent -1.0, -0.5, -0.2, -0.1, 0.0, 0.1, 0.2, 0.5, and 1.0 a.u. (———— negative, — — — zero, – – – – positive).

viously mentioned (p. 61) methylcobaloxime contains a five co-ordinate cobalt(III) in an approximately square-pyramidal environment. The LUMO of $CH_3Co(dmg)_2$ is described approximately as cobalt $3d_{z^2}$ with some admixture of $4p_z$, providing an axial orbital directed towards the vacant site. For convenience the molecule is orientated so that the $Co(dmg)_2$ fragments lie in the xy plane and the y axis bisects the central C-C bond of each (dmg) ligand. The first occuppied orbitals are the d_{zy} and d_{yz} at -12.26 and -12.68eV respectively. When the molecule aquomethylcobaloxime is considered the only important interaction between the water and the $CH_3Co(dmg)_2$ involves a p-orbital on the oxygen of the water and the cobalt $3d_{z^2}$. In the xy section of the electrostatic potential around $CH_3Co(dmg)_2$ (see Figure 25) the asymmetric O-H····O bonds[13] are apparent as well as a large positive region, centred on the cobalt, but extending across the dmg ligands. This large positive region is therefore attractive to incoming nucleophiles. However, in Figure 26, a zx section showed not only the methyl ligand directly bound to the cobalt, but more importantly a rather narrow electrostatic potential channel down which an incoming nucleophile is expected to approach the co-ordinative unsaturated electrophilic centre at the cobalt.

Cobalt Corrin Models

The corrinoid structure of Vitamin B_{12}[12,45] (Photograph 2) was too large; thus for the sake of computational economy the structure was substantially simplified. The system investigated was Species 3, where the Vitamin B_{12} macrocyclic skeleton was retained in the correct oxidation level identical to that of the B_{12} chromophore.

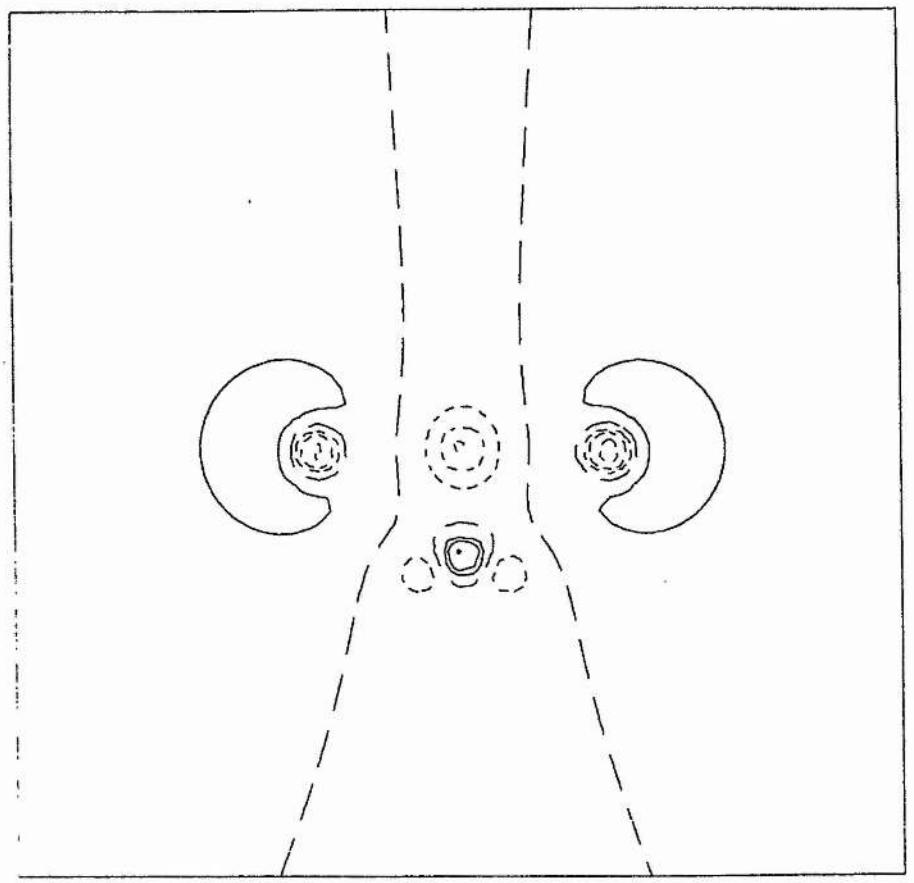
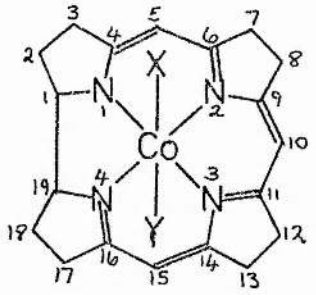


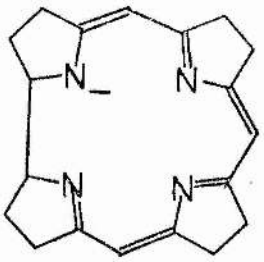
Figure 26 Electrostatic potential in the zx plane of $CH_3Co(dmg)_2$. Successive contours represent -1.0, -0.5, -0.2, -0.1, 0.0, 0.1, 0.2, 0.5, and 1.0 a.u. (——— negative, — — — zero, - - - - positive).

Species 3



All the peripheral ligands on the macrocycle were replaced by hydrogen. The two axial ligands, X and Y, were reserved one for either cyanide or cyanoferrate whilst a range of different substituents were investigated at the second axial site. Initially a series of derivatives was investigated where Y was a series of simple ligands and there was no X substituent. When Y was a good σ donor, the cobalt was calculated to be d^6 Co(III). The d_{z^2} orbital in these complexes was the LUMO. The HOMO was a delocalised π orbital mostly concentrated in the C_{14}-C_{15}-C_{16}-N_4-C_{19} fragment and bonding between C_{14} and C_{15} and between C_{16} and N_4, but otherwise antibonding (Species 3). However, when Y was a good π donor, or absent entirely, the d_{z^2} became the HOMO and the delocalised π orbital the LUMO because in this case the d_{z^2} orbital was lower in energy than the π orbital. This corresponds to a d^8 Co(I) system in which the macrocyclic ligand has undergone a two electron oxidation from Species 4 to Species 5.

Species 4 and 5



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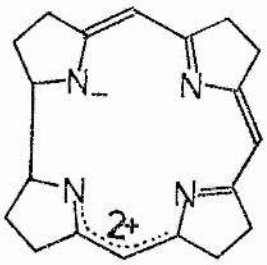

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In summary the system Species 3 was calculated to be Co(III) when Y was NH_3, CH_3^-, or CN^-; but to be Co(I) when Y was absent or was H_2O or NH_2^-. Hence to preserve the oxidation state of cobalt as Co(III) a good σ donor is required as one of the axial ligands. Without such a σ donor an internal reaction between the π electron rich macrocycle and the Co(III) is likely to occur. In the d^8 examples the net charge on the cobalt is always negative, ranging from -0.43e (Y = NH_2^-) to -0.66e (Y = H_2O). In the d^6 cases the net charge on cobalt is always positive, ranging from +0.03e (Y = CH_3^-) to +0.36e (Y = CN^-). Generally, the cyano ligand is thought of as a powerful π acceptor, however the σ donor order deduced from the ligands (Y) studied is $H_2O < NH_2^- < NH_3 < CH_3^- < CN^-$; the cyano ligand is acting as a powerful σ donor. This observation is further confirmed by a recent "ab initio" study of $[Co(CN)_6]^{3-}$ and $[Co(CN)_5OH]^{3-}$. These workers concluded[46] that the principal difference between CN^- and OH^- acting as a ligand arose from their σ donor capacity rather than from their π donor/acceptor properties.

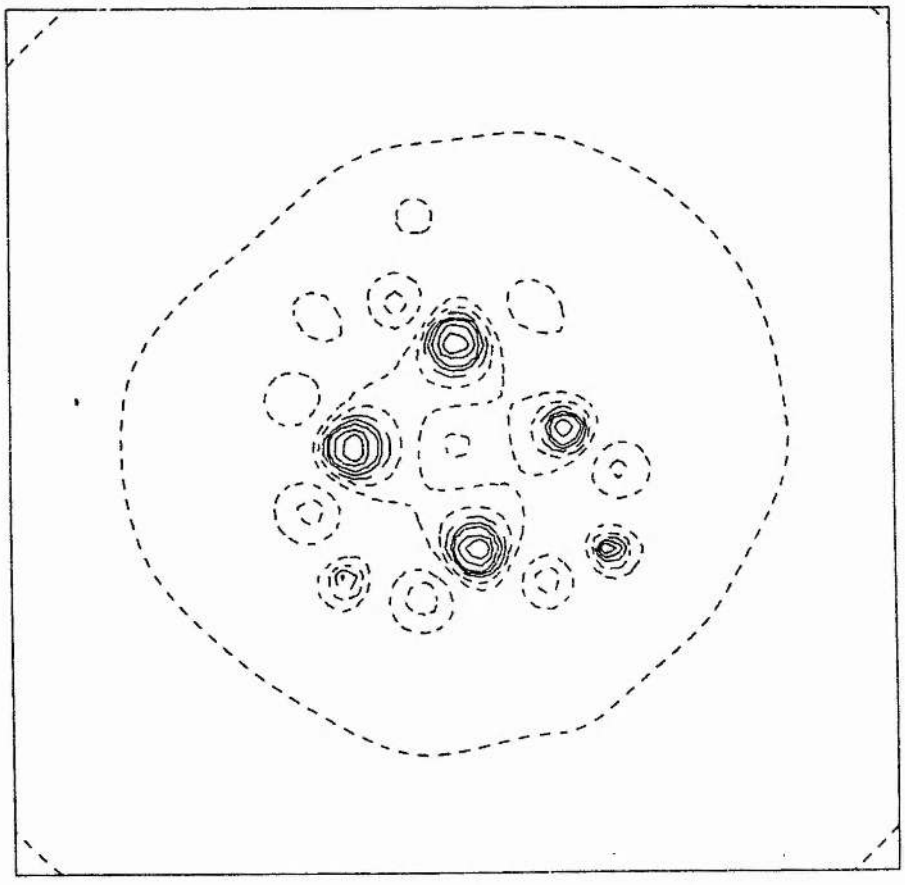

Figure 27 Electrostatic potential of Species 3 (Y = NH_3) in the CoN_4 plane. Successive contours represent -1.0, -0.5, -0.2, -0.1, 0.0, 0.1, 0.2, 0.5, and 1.0 a.u. (———— negative, — — — zero, - - - - - positive).

Plots showing a section of the electrostatic potential for the cobalt corrin model having Y = NH_3 were recorded. Figure 27 is a section through the best plane through the cobalt atom and the four nitrogens. The section illustrates the irregular contour pattern. This is partly due to the deviation of the macrocycle from planarity.[12] The peripheral carbon and hydrogen atoms exhibit differing deviation from the chosen plane for the section. Figure 28 shows a section perpendicular to that shown in Figure 27. This section of electrostatic potential which illustrates the CoN_4 plane is dominated by the uniformly positive residual charges on the peripheral atoms of the macromolecule. This is in contrast to the cobaloxime, $CH_3Co(dmg)_2$ (see Figures 25 and 26) where there are substantial negative regions around the oxygen atoms. The only negative regions of the corrin ring (see Figures 27 and 28) are those in the immediate vicinity of the ligating nitrogens and the quasi-aromatic carbons C_5, C_{10}, and C_{15}, whereas in $CH_3Co(dmg)_2$ the nitrogen atoms all bear a net positive residual charge, consequent upon their being bound to oxygen.

The Methylcobaloxime:Nitroprusside Complex

Certain assumptions had to be made in order to determine by EHMO calculations the interaction between methylcobaloxime and nitroprusside, namely, that the structure of the methylcobaloxime fragment is unchanged from that of the aquo complex.[13] The bond lengths in the nitroprusside fragment were unchanged from the free ion values[4] and all the inter-bond angles around iron set to 90°. The Fe-X-Y-Co bridge was assumed to be linear, although there are cases of modest

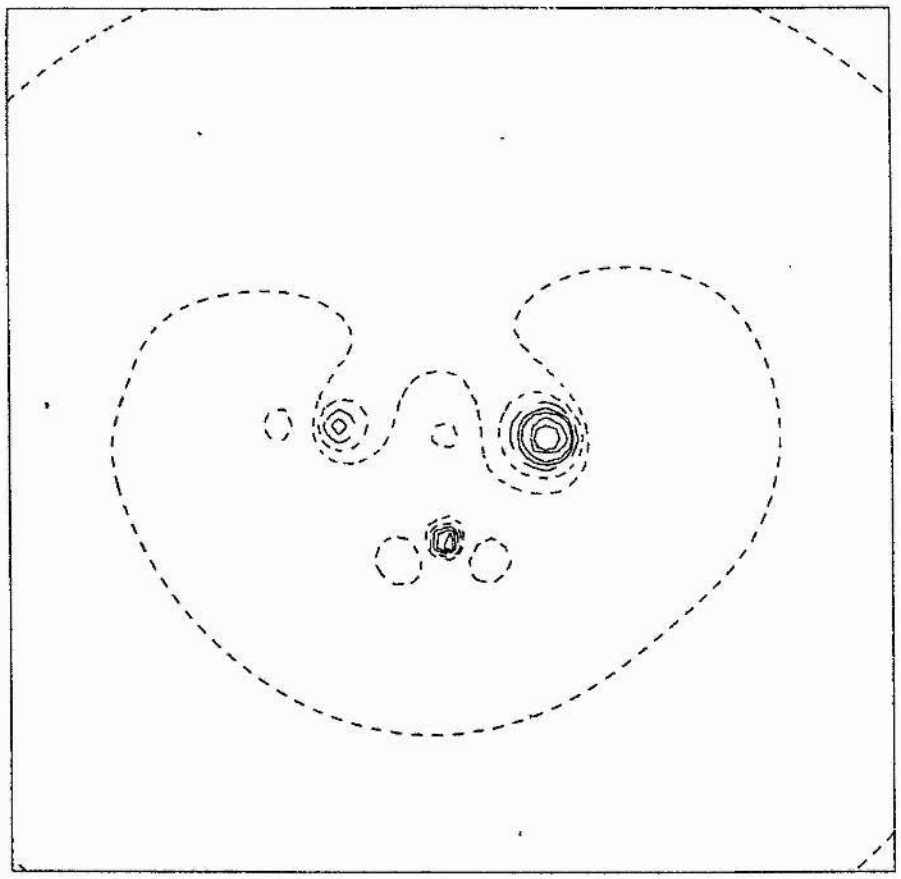

Figure 28 Electrostatic potential of Species 3 (Y = NH_3) in a plane perpindicular to that of the CoN_4 plane. Successive contours represent -1.0, -0.5, -0.2, -0.1, 0.0, 0.1, 0.2, 0.5, and 1.0 a.u. (———negative, — — —zero, - - - - -positive).

deviations from linearity of Co-C-N-Co bridges in the isomeric compounds $[(NH_3)_5Co\text{-}CN\text{-}Co(CN)_5]$[47] and $[(NH_3)_5Co\text{-}NC\text{-}Co(CN)_5]$.[48] These deviations from linearity are a result of forces exterior to the bridges rather than a result of any factor intrinsic to the bridge.

Three isomeric forms were investigated having the nitroprusside bound to cobalt via the axial cyanide, the equatorial cyanide, and the nitrosyl ligands respectively, subject to the above assumptions. All three isomers showed clear energy minima as the distance Y-Co was varied. For the nitrosyl ligand binding to cobalt the O-Co distance for minimum energy was 1.65Å, while for the axial and equatorial cyano ligands binding to cobalt the N-Co distances corresponding to the energy minima were 1.80Å and 1.82Å respectively. Taking the axial cyano isomer as zero, the isomers are +1.9kJ mol^{-1} for the equatorial cyano isomer, and +22.2kJ mol^{-1} for the nitrosyl isomer. Therefore the experimental preference found by NMR for the axial cyano binding in a complex of 1:1 stoichiometry is only very slight. In the case of the axial cyano isomer of the 1:1 complex comparison of the energy at the minimum with the energies of its two components, nitroprusside and methylcobaloxime, shows that the interaction energy equivalent to the axial N-Co bond energy term is 235kJ mol^{-1}. This value does not take into account solvent effects and refers only to an isolated species. Nevertheless, these energies calculated for the isomeric forms are probably reasonably accurate as these solvent effects would be similar in all three isomers.

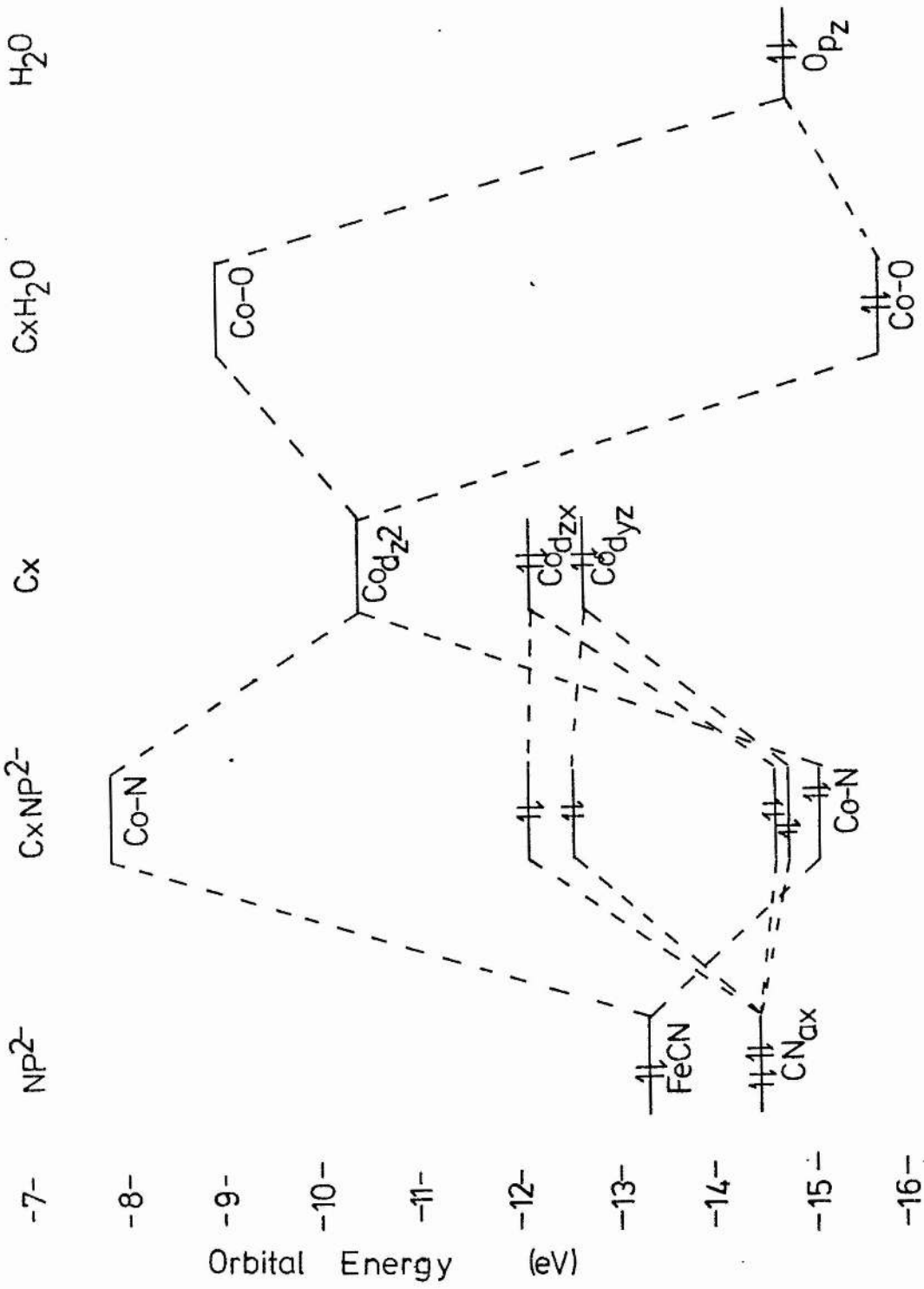

Figure 29 The important orbital interactions between $CH_3Co(dmg)_2$ (Cx) and $[Fe(CN)_5]^{2-}$ (NP^{2-}) or H_2O.

Figure 29 details the principal orbital interactions within the 1:1 complex of nitroprusside and methylcobaloxime. For the axial isomer there is a very strong σ interaction between the axial σ(FeCN) orbital in the nitroprusside (which approximates to a nitrogen lone pair) and the cobalt d_{z^2} orbital, coupled with a very small π interaction involving the axial π(CN) orbitals of the nitroprusside and the cobalt d_{yz} and d_{zx} orbitals, all of which are occupied. Neither in this 1:1 complex nor in the very simple model, trans-$[(ON)Fe(II)H_4CNCo(III)H_5]^{4-}$, is there any interaction between the occupied cobalt d_{yz} and d_{zx} and the unoccupied π^*(CN) orbitals of the axial ligand. Since cyanoferrates readily bind through nitrogen to simple electrophiles such as BF_3 (49-51) and H^+, (51,52) such π back-bonding from the electrophile to the ligand π^* orbitals is not necessary for the stabilisation of cyano-bridged complexes of the type M-C-N-E. Structural evidence in the two linkage isomers of $[(NH_3)_5Co\text{-}CN\text{-}Co(CN)_5]$ (47,48) indicates that even when the appropriate orbitals are present, the π back-binding interaction appears to be negligible in the Co-CN bond as was calculated here, although π back-bonding is significant in the Co-CN bonds.

The Methylcobaloxime:Hexacyanoferrate(II) Complex

The 1:1 complex of hexacyanoferrate(II) and methylcobaloxime is very similar to the cyano bound isomers of the nitroprusside complex with methylcobaloxime. The minimum energy occurs at a N-Co distance of 1.83A, where the interaction energy (N-Co bond energy) is 174kJ mol^{-1}, less than the interaction energy in the $[Fe(CN)_5NO]^{2-}$ complex

by some 60kJ mol^{-1}. In comparing the axial N-Co overlap populations, the hexacyanoferrate(II) complex has a value of 0.297 as compared with 0.309 for the nitroprusside complex (axial isomer). For the binding of nitroprusside via the nitrosyl ligand, the corresponding O-Co overlap population is only 0.257. These calculations are entirely consistent with the previously discussed ^{13}C NMR studies, pointing to a rather weaker binding of hexacyanoferrate(II) compared with nitroprusside. In conclusion for the binding of $[Fe(CN)_5NO]^{2-}$ and of $[Fe(CN)_6]^{4-}$ to methylcobaloxime, the Co-NC distances corresponding to the energy minima are significantly longer than the isomeric M-CN distances. These observations and the absence of any π bonding between cobalt and the bridging cyano ligand are in agreement with experimental results on the isomeric μ-cyano complexes $[(NH_3Co-(CN)Co(CN)_5]$.

The Cobalt Corrin:Nitroprusside Complex

The interaction between nitroprusside and the cobalt corrin model (Species 3) was studied as a function of the axial N-Co distance, subject to essentially the same constraints as mentioned in the methylcobaloxime system. Only the axial isomer of nitroprusside was studied for the sake of computational economy. The energy minimum for the 1:1 complex was located at a Co-N distance of 1.75Å; this is rather shorter than in the cobaloxime complex (1.80Å). This Co-N distance of 1.75Å corresponds to an interaction energy of 288kJ mol^{-1}, substantially higher than for the cobaloxime complex (235kJ mol^{-1}). In the cobalt corrin:nitroprusside complex the orbital interactions involved are very similar to those in the simpler cobaloxime model;

nevertheless, the N-Co overlap population is 0.347 in the corrin system compared with only 0.309 in the cobaloxime complex. Thus, the interaction of nitroprusside is calculated to be much stronger with the cobalt corrin system (Species 3, Y = NH_3) than with a cobaloxime. This reproduces the NMR experimental findings that nitroprusside forms far more stable complexes with cobalamins than with $[CH_3Co(dmg)_2]$. Although all of these cobalt species contain Co(III), in the $[CH_3Co(dmg)_2]$ the cobalt is bound to two uninegative $(dmg)^-$ ligands and to a negative CH_3 axial ligand, whereas in both aquocobalamin and Species 3 (Y = NH_3) the axial ligand is neutral, either a benzimidazole or NH_3 respectively, and the macrocyclic ligand is only uninegative. Therefore on these grounds alone the cobalt in $[CH_3Co(dmg)_2]$ is expected to be rather less electrophilic than in Species 3, or in a natural cobalamin. Nonetheless, the binding of $[Fe(CN)_5NO]^{2-}$ to $[CH_3Co(dmg)_2]$ demonstrates that such complex formation is not a unique property of cobalamins (Vitamin B_{12}).

Further Comparisons between Experimental NMR Studies and EHMO Calculations

In 2:1 Co:Fe complexes observed in NMR experiments, both steric and electrostatic considerations require the two cobalts to be bound to a pair of *trans* ligands in a cyanoferrate. Although the results of a 1:1 complex (nitroprusside:methylcobaloxime) indicate a small energy advantage for the axial bound cyano, the additional binding energy resulting from complexation of the cyanoferrate to a second cobalt species overcomes this small preference for axial binding. Thus, the 2:1 complexes of nitroprusside involve a *trans* pair of equatorial cyano ligands, entirely consistent with the previous ^{13}C NMR studies.

In the case of the $[Fe(CN)_6]^{4-}$ all the ligand sites yield complexes of identical energy. Consequently, there is no thermodynamic impediment to migration of the cobalt species from one cyano ligand to another in these complexes. Such fluxional behaviour in the case of $[Fe(CN)_6]^{4-}$ could explain why the solution state ^{13}C NMR observed for the $[Fe(CN)_6]^{4-}$:cobalamin system contained broad unresolved resonances from which no coupling data could be derived, whereas, in the analogous $[Fe(CN)_5NO]^{2-}$ system very sharp, well resolved first order spectra were always observed.

In conclusion, combined NMR studies and EHMO calculations have shown that the nitroprusside anion binds to aquocobalamin to form both 1:1 and 1:2 nitroprusside:H_2OCb complexes. The nitrosyl ligand is not crucial to this complexing, since hexacyanoferrate(II) also binds to aquocobalamin, although in solution it appears to form a fluxional system. In each case bridges of the type Fe-C-N-Co are proposed since the Co-N-C distances corresponding to the energy minima are significantly larger than the isomeric M-CN distances. Such binding of cyanoferrates also occurs with the simple aquomethylcobaloxime ($[CH_3Co(dmg)_2H_2O]$), but here the binding is very much weaker.

The formation of the 1:1 and 1:2 complexes between nitroprusside and aquocobalamin reported here has been observed at concentrations of nitroprusside that are somewhat higher than those commonly used in clinical practice, these are typically 10^{-5} mol dm^{-1} or below. Therefore, in the succeeding chapter (Chapter 4) the hypotensive in vivo behaviour of these complexes is studied.

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Chapter 4

IN VIVO STUDIES OF SNP/VITAMIN B_{12a} BINDING, AND THE INTERACTION OF SNP WITH OTHER MACROMOLECULES

Abbreviations

SNP = Sodium Nitroprusside

H_2OCb = Aquocobalamin

ST = Sodium Thiosulphate

Hb = Haemoglobin

O_2Hb = Oxyhaemoglobin

MetHb = Methaemoglobin

NAC = N-Acetyl-L-cysteine

BSA = Bovine Serum Albumin

4.1 INTRODUCTION

Aquocobalamin (H_2OCb) has been suggested in the medical literature as an appropriate adjunct during SNP therapy.[1] *In vivo* H_2OCb is thought to prevent successfully, the so-called cyanide release associated with SNP infusion.[2] Others report that, as well as H_2OCb not being an effective antidote to SNP associated cyanide toxicity, it raises the free cyanide levels in the plasma.[3] As a result of the *in vitro* studies previously discussed (Chapters 2 and 3) showing aquocobalamin not only to be a bad antidote to cyanide poisoning but actually to bind to the nitroprusside anion, a series of *in vivo* studies were carried out. These *in vivo* studies were initiated to examine the pharmacodynamic and pharmacokinetic consequences of the 1:1 and 1:2 SNP:H_2OCb complexes discussed in Chapter 3.

A pharmacokinetic study of a drug is the study of the passage of the drug from its point of entry into the body until it reaches its site of action. A study of the interaction of a drug with its site of action in a biological system is a pharmacodynamic study.

The known chemistry of the nitroprusside anion points to the nitrosyl ligand as being the crucial site in the physiological activity of nitroprusside.[4] If the NO is indeed the active site, then the formation of a 1:1 complex with cobalamin will not impair the accessibility of the nitrosyl ligand to external nucleophiles; such as those in potential receptors. In the case of the 2:1 complex with cobalamin (Photographs 4 and 5) the nitrosyl ligand is less accessible due to the corrin macrocycles. The hypotensive site of action is

still under investigation and will be discussed later in this chapter. However, if the anion requires access from the blood stream to its receptor via a specific ion channel through a membrane, e.g. the chloride or calcium channel, then formation of even the 1:1 complex may hamper such access through the membrane. Therefore, *in vivo* studies were carried out initially to investigate these hypotheses.

The administration of sodium thiosulphate (ST) is thought to reduce the toxic effects of SNP.[5,6] Baumeister[7] reported that exogenous ST replenishes depleted endogenous ST stores used up by initial rhodanase (thiosulphate cyanide sulphur transferase EC 2.8.1.1)- mediated cyanide detoxification. Schulz[8] claimed that in order to retain the pharmacological benefits of SNP but remove the possibility of cyanide toxicity, a "mixed infusion" technique was preferable, in which ST and SNP, in a 12:1 molar ratio, are mixed in the infusion fluid prior to injection. Therefore, in the present study the pharmacological consequences of mixing ST and SNP were investigated *in vivo* and compared with the pharmacological effects of mixing SNP and H_2OCb.

Although the nitroprusside anion when infused into the blood stream is totally soluble in the plasma, these *in vitro* studies showing SNP binding to cobalamin suggest that it might also interact with other components of whole blood. Initially the *in vitro* interaction of nitroprusside and the universal protein carrier serum albumin was studied by Sephadex G-25 column chromatography. Then the *in vitro* investigation of different types of haemoglobin with SNP was studied by such techniques as electrophoresis, uv-visible spectroscopy, ^{13}C

and ^{15}N NMR, and EPR spectroscopy. These *in vitro* studies are of importance in understanding the *in vivo* hypotensive action of nitroprusside. SNP is thought to undergo a *trans*-nitrosylation reaction[9] with the enzyme guanylate cyclase (GTP pyro- phosphate lyase (cyclising) EC 4.6.1.2). The mechanism is still a very controversial subject[10-12], but it has been established that the enzyme produces guanosine-3',5'-monophosphate[13] (cyclic GMP) which is involved in the vascular smooth muscle relaxant response to nitrogen-oxide containing drugs.[14] Some workers[15] have reported that the initial enzyme activation requires the presence of activated thiol groups in the form of an unstable short lived S-nitrosothiol[16], e.g. S-nitrosocysteine, and that it is the NO^+ which is the vascular smooth muscle relaxant. However, recent observations[12] have shown that partially purified hepatic guanylate cyclase readily binds haem and NO-haem which suggests that haem may also be related to the mechanism of guanylate cyclase activation by NO^+ and that haemoproteins are required to restore guanylate cyclase activation in response to NO^+. The formation of NO-haem complexes[17] which bind to[18] and activate[19] guanylate cyclase is suggested by Ignarro[12] as being the likely reason for the requirement of haem as well as NO^+. This present work studies in more depth the *in vitro* interaction of nitroprusside with different forms of haemoglobin.

4.2 EXPERIMENTAL

4.2.1 PHARMACOLOGICAL ASPECTS OF THE INTERACTION OF NITROPRUSSIDE WITH AQUOCOBALAMIN AND THIOSULPHATE

I am indebted to Dr D.S. Hewick and his collegues of the Department of Pharmacology and Clinical Pharmacology at Ninewells Hospital and Medical School for carrying out all the *in vivo* experiments reported herein.

Materials

As in Chapter 3 (p. 42) aquocobalamin was purchased from Sigma. Sodium thiosulphate was of AnalaR grade. The sodium nitroprusside-^{14}C used here is part of the sample prepared previously as detailed in Chapter 2 (p. 17). All solutions of aqueous SNP were carefully protected from light.

Animals

For these *in vivo* studies male Sprague dawley rats (450-650g), maintained on a normal diet with free access to water, were used.

Measurement of Blood Pressure and Depressor Response

The rats were anaesthetised with urethane (1.5^{-1}Kg ip) and the rectal temperature maintained at $37^{o}C$ by means of a heat lamp reg-

ulator device. In each rat the right artery and left jugular vein were cannulated for measurement of blood pressure and injection of drugs respectively. The carotoid cannula was connected to a blood pressure transducer (Elcomatic Ltd.) and the blood pressure was monitored on a recorder. The venous cannula was connected to a 1.0ml syringe. The drug solutions were made up in physiological saline, in a volume of 0.1-0.3ml and washed in with 0.1ml saline.

Separate comparisons were made between SNP and a SNP:H_2OCb mixture, or SNP and a SNP:ST mixture. For each comparison a high (A) and low (B) dose of SNP were used along with a high (C) and low (D) dose of the mixture. In each case the low dose was 25-50% of the high dose. During the comparison, in order to allow for any sensitivity changes in the preparation during the course of the experiment, each dose was given four times in a random order; i.e. (ABCD), (CABD), (BCDA), (ACDB).

The hypotensive action which is measured by depression response was assessed in the following ways;-

(i) blood pressure lowering - the difference between the mean systolic pressure before audition of the drug and the lowest systolic pressure after drug addition,

(ii) "onset-time" - the time between drug addition and maximum blood pressure lowering,

(iii) "offset-time" - the time between maximum blood pressure lowering and normalisation of blood pressure.

Measurement of Plasma Concentrations of SNP-^{14}C Derived Radioactivity

The rats were anaesthetised and cannulated as previously described, the artery and vein being used for withdrawal of blood and injection of drugs respectively. Sodium nitroprusside ($5\,\mu mol^{-1}$ Kg, $0.3\,\mu Ci^{-1}$ Kg) was injected in physiological saline, either alone or with aquocobalamin or sodium thiosulphate, and washed in with saline. At 1, 2, 4, 6, 10, 20, and 40 minutes blood samples (0.4ml) were withdrawn and centrifuged (3000g for 10 minutes) to obtain plasma. These plasma samples (0.1ml) were transferred to a 5ml NE 260 scintillator for scintillation counting. The plasma radioactivity was expressed as SNP equivalents. Control experiments were carried out daily using two rats; one receiving SNP alone and one receiving a mixture of SNP with H_2OCb or ST. The results are expressed as means ± se. Students t-test was used for comparisons, coupled with the paired t-test being used for comparisons within the same animal. A probability of < 0.05 was taken to be significant.

4.2.2 IN VITRO INTERACTION OF SNP AND BOVINE SERUM ALBUMIN

Materials

Bovine serum albumin, (BSA, fatty acid free) and Sephadex G-25 were purchased from Sigma. Malononitrile and sodium nitroprusside were of AnalaR grade. Chromatographic columns were eluted using isotonic buffer, pH 7.2, prepared as previously mentioned (Chapter 2,

p. 18). At every stage of analysis all aliquots of SNP were carefully protected from light.

Sephadex G-25 Column Chromatography

Initially SNP (0.01g, 3.3×10^{-5}mol) was added to a solution of bovine serum albumin (0.6g, 9.05×10^{-5}mol) in isotonic buffer and incubated for one hour at 37°C. The resulting mixture was added to a Sephadex G-25 column (30 x 2cm) and eluted with isotonic buffer over a period of four hours, protected from light at all times. Aliquots (5ml) of solution were individually collected and analysed for albumin and SNP content.

Albumin and SNP Determination

Each 5ml aliquot eluted from the chromatography column was initially analysed for bovine serum albumin content. This was done by recording the UV absorption at λ_{max} 280nm on a SP8-100 spectrophotometer at 37°C. Proteins show a characteristic absorption at this wavelength λ 280nm. (In the case of albumin this absorption maximum is due to the presence of tyrosine and tryptophan amino acid residues.) Each aliquot was then analysed for nitroprusside content. The carbanion of malononitrile reacts with nitroprusside to form a highly coloured adduct[20], and this proves to be an excellent spot test for SNP. A solution of malononitrile (1.0×10^{-3}mol dm^{-3}) at pH 11.5 was prepared and 1ml volumes were added to 1ml of the eluted aliquots. This spot test was completed within one hour, since the alkaline solution of preformed malononitrile carbanion is only stable for a few

hours. The presence of albumin in the aliquots did not interfere with the determination of SNP content, since a control experiment of an alkaline solution of albumin and malononitrile proved to be negative; no highly coloured species was formed under these conditions.

The whole *in vitro* study and analysis was carried out three times and gave consistent results each time.

^{13}C NMR Spectrum of SNP-^{13}C in the Presence of Bovine Serum Albumin

To an saturated solution of albumin in isotonic buffer, pH 7.2 (10% D_2O) was added 90% SNP-^{13}C (0.05mol dm^{-3}). The spectrum was recorded on a Varian CFT-20 instrument with an accumulation of 3000 scans at 25°C, a pulse width of 7μsec and a pulse delay of 0sec.

4.2.3 IN VITRO INTERACTION OF SNP WITH HAEMOGLOBINS

Materials

Bovine haemoglobin, methaemoglobin, bovine haematin, D,L-dithiothreitol, aquocobalamin, and glutathione (reduced form) were all purchased from Sigma UK. L-cysteine hydrochloride, N-acetyl-L-cysteine (NAC), and sodium dithionite were of AnalaR grade and purchased from Aldrich. Sodium nitroprusside and sodium hexacyanoferrate(II) were of AnalaR grade and purchased from BDH. ^{13}C-labelled nitroprusside and hexacyanoferrate(II) were prepared as previously mentioned (Chapter 3, p. 42).

Extraction of Human Haemoglobin

A blood sample was extracted with a small scalpel. The whole blood sample was then left to coagulate. The resulting sample was centrifuged (3000 r.p.m. for 30 minutes) after which the plasma was decanted off. The bolus obtained was treated with acetone to lyse the erythrocytes. The haemoglobin samples were used within two hours of extraction.

Preparation of Bovine Oxyhaemoglobin

This is an adaption of a well established method.[19] The required quantity of bovine methaemoglobin was converted to oxyhaemoglobin by dissolving the MetHb in isotonic buffer, pH 7.2, and adding dropwise a solution of sodium dithionite (0.1mol dm^{-3}). The conversion to O_2Hb was monitored by uv-visible spectroscopy using an SP8-100 spectrophotometer. The O_2Hb Soret band is at 412-415nm and the appearance of this band was monitored.

Preparation of Bovine Deoxyhaemoglobin

An isotonic buffered solution of bovine MetHb was purged with nitrogen for three hours to remove all the oxygen, and treated with sodium dithionite (0.1mol dm^{-3}) added dropwise. The uv-visible spectrum was monitored until the Soret band at 430nm appeared.

Preparation of Haem

This is an adaption of an established method.[12] To a solution of phosphate buffer, pH 7.6, is added the required amount of haematin and the solution stirred in an ice/salt bath until the temperature had equilibrated at 0-4°C. To this was added a ten-fold excess of dithiothreitol and the resulting solution stirred at 0-4°C for a further 30 minutes. The brown solution was then extracted exhaustively with petroleum ether (60-80) to remove unreacted dithiothreitol. The removal of dithiothreitol was monitored by spot testing with solutions of SNP.

Electrophoresis Studies

A Gelman 1 electrophoresis tank was employed for these studies. The chambers of the electrophoresis apparatus were filled with 500ml of barbitone buffer, pH 8.6. Cellulose acetate strips were used as the electrophoresis support medium. For optimum results solutions (1.62×10^{-5} mol dm^{-3}) of each type of haemoglobin was prepared in isotonic buffer, pH7.2, and used as controls. Samples of Hb (1.62×10^{-5} mol dm^{-3}), and SNP (3.2×10^{-6} mol dm^{-3}) (i.e.> 5:1 Hb:SNP) were prepared and carefully protected from light. Each control Hb and its corresponding Hb:SNP mixture were applied to two independent cellulose acetate strips. This ensured no spurious results due to differences in the acetate strips. The electrophoresis process took one hour after which the cellulose acetate strips were developed in coomassie blue dye.

UV-Visible Spectroscopy Studies

All spectra were recorded on a Perkin-Elmer λ_B spectrophotometer, with computer spectra subtraction facility. Concentrations varied depending upon the exact stoichiometries employed, but haemoglobins and aquocobalamin concentrations were usually 1.62 x 10^{-5}mol dm^{-3} and 2.1 x 10^{-4}mol dm^{-3}.

^{13}C and ^{15}N NMR Studies

All ^{15}N NMR spectra were recorded in the FT mode at 25°C on the Bruker WH-360 spectrometer. The ^{15}N spectra were recorded at 36.5MHz relative to $CH_3{}^{15}NO_2$ as the external reference with spectral widths of 15151Hz, with a pulse delay of 1.08s between pulses of 5-9 seconds.

Sodium pentacyano(^{15}N)nitrosylferrate(II) was prepared as described previously[21], then recrystallised exhaustively from methanol to remove excess nitrile. To solutions of $Na_2[Fe(II)(CN)_5{}^{15}NO_2]$ (0.1mol dm^{-3} and 5.75 x 10^{-2}mol dm^{-3}) in phosphate buffer, pH 7.6 (10% D_2O), in successive experiments were added;- (i) haem (5.75 x 10^{-2}mol dm^{-3}), (ii) N-acetyl-L-cysteine (0.1mol dm^{-3}), and (iii) N-acetyl-L-cysteine (5.75 x 10^{-2}mol dm^{-3}) and haem (5.75 x 10^{-2}mol dm^{-3}).

Preliminary ^{13}C NMR spectra were recorded on a Varian CFT-20 spectrometer. The spectra were recorded at 20MHz relative to external TSPSA. The number of scans was typically 2000-4000, using a pulse delay of 0sec and a pulse width of 7μsec. Generally the concentra-

tions of 90% ^{13}C-labelled cyanoferrates in 10% D_2O phosphate buffer, pH 7.6, or isotonic buffer, pH 7.2, were 0.05mol dm^{-3}. Natural abundance ^{13}C NMR spectra of the cyanoferrates were generally run at 1mol dm^{-3} in 10% D_2O isotonic buffer. Concentrations of the haemoglobins, sodium dithionite, and dithiothreitol varied depending upon the exact stoichiometries employed.

90Mhz NMR spectra were recorded in the FT mode on the Bruker WH-360 spectrometer of the S.E.R.C. NMR service at the University of Edinburgh. All spectra were recorded at 25°C relative to external TMS using spectral widths of 16129Hz and typically requiring 5000 scans with pulse delays of 1.01s between pulses of 4 μs. Concentrations of 90% ^{13}C-labelled SNP in 10% D_2O phosphate buffer ph 7.6, or isotonic buffer pH 7.2 were 0.05mol dm^{-3}.

EPR Spectroscopy Studies

EPR studies were carried out at 298K in quartz capillaries using a Bruker ER 200D spectrometer. Di-t-butyl nitroxide was used as the standard for the measurement of the line positions. Concentrations of SNP were typically 0.5mol dm^{-3}, concentrations of thiols, dithionite, and dithiothreitol were 0.05mol dm^{-3} in phosphate buffer, pH 7.6. Concentrations of deoxyhaemoglobin and haem were 0.01 mol dm^{-3} and 0.1mol dm^{-3} respectively in isotonic buffer, pH 7.2 and 7.6. I would like to thank Dr J.C. Walton the use of the EPR spectrometer, and Mr I. Johnson for his help and advice.

Molecular Computer Graphics

All atomic parameters and structural data were obtained from the brookhaven Protein Data Bank and Protein Sequence Query System (Daresbury) provided by the S.E.R.C. As discussed previously (Chapter 3, p. 46) the program ChemX in conjunction with VAX 11/785 and Tektronics hardware were employed for subsequent molecular modelling and graphical display.

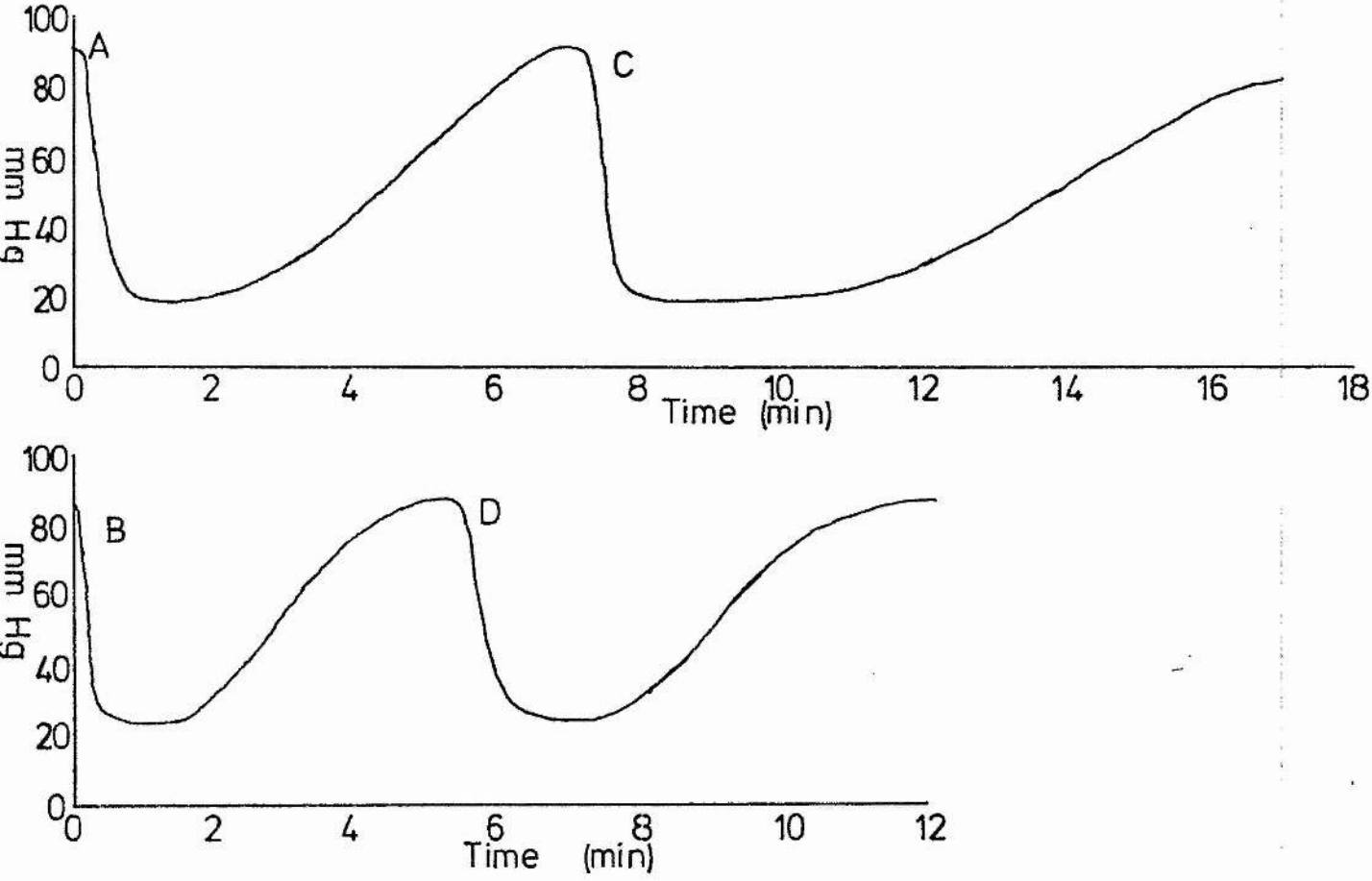


Figure 1 Blood pressure depressor response plots measuring the effect of 10:1 H_2OCb:SNP maximal doses.

Dose A 6×10^{-7} mol SNP

C 6×10^{-7} mol SNP and 6×10^{-6} mol H_2OCb

B 2×10^{-7} mol SNP

D 2×10^{-7} mol SNP and 2×10^{-6} mol H_2OCb

4.3 RESULTS AND DISCUSSION

4.3.1 PHARMACOLOGICAL ASPECTS OF THE INTERACTION OF NITROPRUSSIDE WITH AQUOCOBALAMIN AND THIOSULPHATE

The Effects of Aquocobalamin on the Pharmacodynamics and Pharmacokineties of Sodium Nitroprusside

In Chapter 2 preliminary experiments using SNP-^{14}C and uv-visible spectroscopy studies, undertaken to examine the stability of SNP in blood and buffer suggested that SNP forms a complex with aquocobalamin. Further studies using high field NMR spectroscopy (Chapter 3) showed that on mixing aquocobalamin and SNP in 1:1 and 10:1 or 2:1 molar ratios resulted in 1:1 and 2:1 cobalamin:nitroprusside complexes respectively. The ^{13}C NMR spectra coupled with EHMO calculations demonstrated that the formation of the complexes involved Fe-C-N-Co bridges, where the nitrogen atom of the cyano ligand of SNP is directly co-ordinated to the cobalt atom of the cobalamin. Therefore, a series of *in vivo* studies were initiated to examine the pharmacodynamic and pharmacokinetic consequences of these 1:1 and 2:1 complexes during the drug action of SNP.

When H_2OCb was added to SNP in a 10:1 or 1:1 molar ratio this caused a significant delay in both the onset and offset of the depressor response to maximal doses (600 or 200nmol) of SNP (see Figure 1). However, the degree of blood pressure lowering achieved was not affected by the presence of H_2OCb in both molar ratios (see Table 1).

TABLE 1 The effect of aquocobalamin on the depressor response to sodium nitroprusside

Molar ratio of H_2OCb: SNP mixture	Dose of SNP (nmole)	Blood pressure lowering (mmHg)			Onset time (sec)			Offset time (sec)		
		SNP alone	Mixture	% change	SNP alone	Mixture	% change	SNP alone	Mixture	% change
	600	64.8 ± 8	68 ± 4	↑ 8	65 ± 7	100 ± 5	↑54*	362 ± 11	444 ± 14	↑23*
10:1	200	56 ± 6	58 ± 6	↑ 4	47 ± 3	67 ± 3	↑43*	278 ± 9	332 ± 13	↑19*
	600	41 ± 1	41 ± 1	0	62 ± 3	64 ± 10	↑ 3	368 ± 22	521 ± 45	↑42*
1:1	200	36 ± 2	36 ± 2	0	45 ± 2	60 ± 5	↑33	254 ± 8	339 ± 9	↑33
	600	38 ± 6	34 ± 4	↓11	47 ± 9	42 ± 12	↓11	398 ± 23	433 ± 25	↑ 9
0.5:1	200	34 ± 5	33 ± 5	↓ 3	41 ± 7	41 ± 4	0	273 ± 25	288 ± 20	↑ 5

Intravenous bolus doses of SNP were given alone or as mixtures with H_2OCb. Each dose was administered four times in the order indicated in "Materials and Methods". The data are representative, one rat being used for each different H_2OCb: SNP mixture. Means ± s.e. are given (n=4). Arrows indicate an increase/decrease compared with SNP alone and asterisks indicate that the difference is significant ($P<0.05$).

Using a 10:1 molar ratio H_2OCb:SNP mixture a similar pattern was obtained with smaller and more importantly established submaximal doses of SNP (20 and 5nmol). In Table 2 the blood pressure lowering, onset and offset values for SNP and the 10:1 mixture for both submaximal doses of 20 and 5nmol of SNP are summarised. Similar

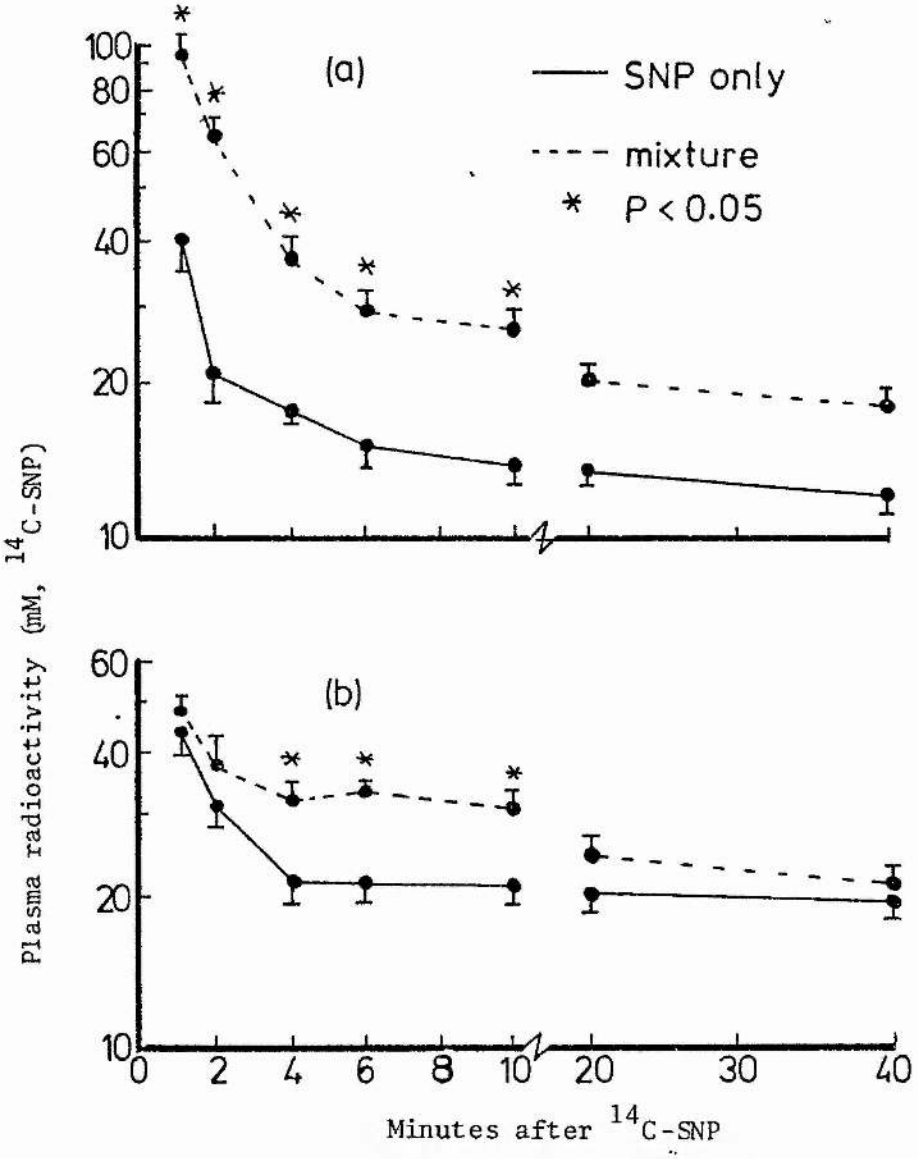
Table 2

Blood pressure lowering, onset, and offset times as a result of submaximal doses of 20nmol and 5nmol of SNP and corresponding 10:1 H_2OCb:SNP mixtures.

Submaximal Dose	Blood Pressure (mm Hg)	Onset Time (s)	Offset Time (s)
SNP alone (20nmol)	20 ± 0.3	26 ± 2	71 ± 4
H_2OCb:SNP 10:1 (20nmol)	18 ± 0.9	50 ± 7	85 ± 4
SNP alone (5nmol)	16 ± 2	22 ± 2	76 ± 4
H_2OCb:SNP 10:1 (5nmol)	14 ± 1	37 ± 3	99 ± 7

results were obtained for two other rats subjected to the same 20 and 5nmol SNP doses alone, or mixed with H_2OCb. Control experiments were carried out using H_2OCb alone which had no hypotensive effect when administered in doses ranging from 50 to 6000nmol. When the H_2OCb was added to SNP (600 or 200nmol) doses in a 0.5:1 molar ratio, it had no significant effect on the depressor response to SNP (see Table 1). In

Figure 2 Plot of Plasma Radioactivity Verse Time After ^{14}C SNP Injection.



The effect of mixing aquocobalamin (a), or thiosulphate (b) with sodium nitroprusside-^{14}C on plasma radioactivity. The aquocobalamin or thiosulphate (10 or 12 molar excess respectively) were mixed with the nitroprusside prior to injection. The nitroprusside dose was 5 mol kg^{-1}, 0.3 Ci kg^{-1} (i.v.). The continuous and dashed lines correspond to "nitroprusside alone" and "mixture" respectively. The data points are means $\pm$ s.e. (n = 4 and 6 for aquocobalamin and thiosulphate experiments respectively). Asterisks indicate a significant difference ($P < 0.05$) from nitroprusside alone.

analysing the data it can be noted that mixing SNP with a molar excess of H_2OCb prolonged the depressor response in anaesthetised rats by 30-70%. However, the addition of H_2OCb appeared not to increase the degree of blood pressure lowering.

When aquocobalamin was added to the injected solution, in a 10:1 H_2OCb:SNP molar ratio, this resulted in an elevation of SNP-^{14}C derived radioactivity in the plasma. The plasma elimination profiles (Figure 2a) for SNP alone and the mixture were roughly parallel during the first 10 minutes of infusion, while concentrations of radioactivity associated with the mixture were 2-3 times greater. Therefore, the prolongation of response noted earlier could be linked with a marked elevation of plasma SNP-^{14}C derived radioactivity when H_2OCb and SNP were injected together in a 10:1 molar ratio mixture. In such a mixture the 2:1 cobalamin:nitroprusside complex will be present. Therefore, it is apparent that such a species tends to retain the infused nitroprusside within the plasma space. If it is assumed that the rat plasma volume is 40.4ml Kg^{-1} (22), it can be concluded that at one minute after injection of the H_2OCb:SNP mixture almost 80% of the injected radioactivity is retained in the plasma, whereas for the injection of SNP alone 31% of the radioactivity is retained in the plasma.

In the case of the 1:1 molar ratio H_2OCb:SNP at established submaximal doses of SNP there appeared to be no delay in the depressor response. Since such a mixture results in the production of a 1:1 cobalamin:nitroprusside complex it is apparent that such complex formation does not impair the mobility of the SNP significantly and

the accessibility of the nitrosyl ligand to the receptor site, whereas at maximal doses (600 or 200nmol) this 1:1 complex causes significant prolongation in both onset and offset of the depressor response. This cannot be easily explained.

In the 10:1 molar ratio of H_2OCb:SNP producing a 2:1 cobalamin: nitroprusside complex, despite the hindrance of the two Vitamin B_{12a} moieties there was no reduction of the hypotensive activity of the SNP. The complex may act as a sort of depot in the plasma. It seems unlikely to be able to gain access to the site of action in the smooth muscle membrane, e.g. guanylate cyclase, to exert any spasmolytic effect. The mode of action of SNP is discussed later in the Chapter, but it can be noted from Photographs 16 and 17 that there is a reasonable area of space for the NO ligand to interact with L-cysteine, thought to be an integral part of the receptor site of guanylate cyclase. Alternatively, the complex could act as a depot, releasing the nitrosyl - the active vasodilator component more slowly than SNP alone.

Hobel and Raithelhuber[23] reported that when SNP-^{14}C doses of 1.3μmol Kg^{-1} were infused into rats, the radioactivity in the blood declined rapidly in the first 4-6 minutes and then fell more slowly. These reports are in general agreement with the work discussed here. However, because of the relatively low specific activity of the SNP-^{14}C used here, the dose of the drug used for the pharmacokinetic studies was about five times higher than the highest dose used for the pharmacodynamic studies. Although it could be seen that the hypotensive effect was associated with a very marked fall in SNP-^{14}C derived

radioactivity in the plasma, it is difficult to relate them precisely. During the first 4-6 minutes, presumably the fall in concentration of the plasma radioactivity is associated with a redistribution of the drug to its site of action in the vascular smooth muscle. Nitroprusside can cause a fall in peripheral resistance and increase the arterial capacity to any desired level within 30 seconds: thus the duration of its action is extremely short, and on discontinuation of infusion, the blood pressure returns to normal usually within 2-5 minutes. Hence, the radioactive SNP-^{14}C after this period will then be metabolised and excreted.

The Effect of Sodium Thiosulphate on the Pharmacodynamics and Pharmacokinetics of Sodium Nitroprusside

When sodium thiosulphate was added to SNP and given as an i.v. bolus dose to the rats, it had no significant effect on the depressor response of nitroprusside. Submaximal doses of 12 and 6nmols of SNP either alone, or as an ST:SNP mixture at 12:1 molar ratio were given to the rats. Saunders and Himlich[24] concluded that the optimum thiosulphate to cyanide molar ratio for rhodanase-mediated detoxification is 2-3:1. However, the fact that each molecule of SNP contains five cyanide ligands leads to the rationale in this present study for using a 12:1 molar ratio mixture. The blood pressure lowering, onset, and offset time values corresponding to the higher and lower doses (12nmol and 6nmol) of SNP and the mixture are shown in Table 3.

Table 3

Submaximal Dose	Blood Pressure (mm Hg)	Onset Time (s)	Offset Time (s)
SNP alone (12nmol)	32 ± 2	22 ± 2	59 ± 3
ST:SNP 12:1 (12nmol)	34 ± 3	23 ± 2	50 ± 4
SNP alone (6nmol)	21 ± 1	20 ± 1	46 ± 7
ST:SNP 12:1 (6nmol)	25 ± 3	19 ± 1	50 ± 4

Consistent results were given by a further three rats. These results initially appear to disagree with the study of Ivankovich et al.[25] They reported that during a five hour infusion in dogs, the dose of SNP alone, unlike that of an 18:1 molar ratio ST:SNP mixture, had to be steadily reduced to maintain a constant level of hypotension. Ivankovich explained this necessary dosage reduction with SNP alone is due to decrease in cardiac reserve due to the toxicity of accumulating cyanide. However, Schulz et al[5] reported that no such indirect enhancement of the hypotensive effect of SNP given "chronically" was detected. They reported that in a single patient, infusion of a 12:1 molar ratio of ST:SNP mixture was equally or slightly more effective than just SNP for lowering the blood pressure. In the present study any effect of cumulative cyanide cardiotoxicity would not be apparent from mean data, using single bolus doses of SNP or ST:SNP mixture given in a randomised order.

However, when individual responses in each rat were examined they showed no increase in the efficancy of SNP during the period of each experiment. This indicates that no cyanide induced cardiotoxicity had occurred.

Addition of sodium thiosulphate to the injection solution to give a 12:1 ST:SNP-^{14}C molar ratio had a less marked influence on the plasma SNP-^{14}C derived radioactivity than H_2OCb (see Figure 2b). The major effect was an elevation of plasma radioactivity at 4, 6, and 10 minutes, of some 50-60%. Since the pharmacological response to SNP was unaffected, it is presumed that the higher concentrations of radioactivity were due to inactive degradation products rather than the active species itself. Hobel and Raithelhuber[23], using SNP-^{14}C of specific activity several hundred times greater than the present study, examined some of the degradation products in the blood. The reported proportions of SNP, cyanide, and thiocyanate in blood at 1, 10, and 40 minutes respectively were 92, 6, and 2%; 68, 25, and 6%; and 28, 30, and 40%.

In comparing ST with the use of aquocobalamin as an adjunct to SNP treatment, H_2OCb suffers from a number of disadvantages. Aquocobalamin has a relatively high molecular weight, so that large amounts of it are required. For example, about 2.5g of H_2OCb would be required to neutralise all the cyanide contained within 100mg of SNP, the recommended dose reported by Cole.[26] Additional disadvantages of H_2OCb, apart from a greater intrinsic toxicity, are a lower antidotal efficacy compared with ST as reported by Hobel *et al.*[27] The present *in vivo* studies show that aquocobalamin and nitroprusside chemically

interact resulting in a prolonged and probably less predictable pharmacological response. The mechanism of vasodilatory action still requires further investigation.

4.3.2 THE IN VITRO INTERACTION OF NITROPRUSSIDE AND BOVINE SERUM-ALBUMIN

Albumin has a relatively high concentration in the plasma (0.68mmol dm^{-3}). It plays an important role in regulating colloid-osmotic pressure. Since the isoelectronic point of albumin is 4.9, it occurs in the blood predominantly in anionic form and so can bind cations. On the other hand, it can also bind anions, and in this way manifests a type of detergent function. In this respect albumin can be called a "universal carrier". It is possible that SNP, when infused into the body by i.v. injection, though soluble in the plasma, may in part bind to albumin as a possible means of transport of the drug to the site of action. To investigate any interaction between nitroprusside and albumin, *in vitro* studies were carried out using Sephadex G-25 column chromatography. Initially a 3:1 BSA:SNP mixture was allowed to incubate at 37°C for one hour in a minimum volume of isotonic buffer pH 7.2, then the resulting solution was added to a Sephadex column and eluted with isotonic buffer. Small aliquots were individually collected and analysed for both nitroprusside and albumin content. The results of the indicated analysis a small quantity of protein was eluted first from the column but all of the SNP and the majority of the BSA were eluted together. Therefore,

this is definite evidence of an interaction between SNP and the protein that can withstand elution through an anionic column. In an effort to investigate further this interaction a ^{13}C NMR spectrum was recorded of 90% ^{13}C-labelled SNP and BSA mixture at the same 3:1 protein:SNP ratio. The 20MHz ^{13}C NMR spectrum showed the characteristic second order SNP resonance. This indicates that there is neither an ionic nor covalent interaction between SNP and BSA. However, to withstand elution down a Sephadex column, there must in fact be a hydrogen bonding interaction, which has no significant effect on the 90% ^{13}C enriched spectrum of SNP. This type of interaction can be explained if the detergent function of albumin is considered; when anions are bound to albumin this involves a hydrogen bonding interaction.

4.3.3 IN VITRO INTERACTION OF SNP WITH HAEMOGLOBINS

Since SNP binds so readily to aquocobalamin (Vitamin B_{12a}), its model compound, aquomethylcobaloxime, and albumin it may interact with other macromolecules concentrated in the blood when it is infused into the blood stream, e.g. haemoglobin (see Photograph 18), a protein composed of four peptide chains (2 α and 2 β subunits, see Appendix 2). Each subunit contains a haem moiety, a porphyrin ring system (see Photograph 19). This is a very controversial subject, since it has been argued in the literature[12,28] for several years that nitroprussides mode of vasodilating action involves the formation of nitrosyl-haemoglobin by interaction with haemoglobin.

It is this species which activates the thiol groups of the enzyme guanylate cyclase (EC 4.6.1.2) whose primary product cyclic-GMP is implicated in the direct action of vascular smooth muscle relaxation. While Baughler and others[10,11,29] refute the requirement for haem, they instead claim that the nitrosylating species, e.g. SNP, reacts with an electrophilic thiol compound, e.g. L-cysteine, to form a short lived S-nitrosothiol, e.g. S-nitroso-L-cysteine, and it is this species which activates guanylate cyclase. Therefore, with this clinical significance in mind the interaction of SNP with various types of bovine haemoglobin and human haemoglobin was initially studied by the technique of electrophoresis.

Samples of 5:1 Hb:SNP at physiologically relevant concentrations were prepared with various types of haemoglobin, e.g. bovine met-haemoglobin, deoxy, and oxyhaemoglobin, and human haemoglobin extracted from fresh blood. These mixtures, and control Hb were applied to two independent acetate strips and subjected to an electrophoresis current. Any interaction of SNP and haemoglobin would cause the mobility of the haemoglobin in the current to be altered in comparison to the control haemoglobin, arising from charge differences between species. The electrophoresis technique showed no differences in mobility between the various haemoglobins; met, oxy, and extracted human haemoglobin and their corresponding 5:1 Hb:SNP samples. In the case of deoxyhaemoglobin there was a slight difference between the control and the 5:1 Hb:SNP sample. This was reproducible, but its significance at this stage could not be assessed further using this technique.

UV-Visible Spectroscopy Studies

In an attempt to investigate these electrophoresis observations, samples of haemoglobins and Hb:SNP discussed previously were studied by uv-visible spectroscopy utilising a computerised spectral subtraction facility. Initially the visible spectrum of the various haemoglobins (1.62×10^{-5}mol dm^{-3}) in the region 400-600nm were recorded (see Table 4). These were then compared with the spectra of the 5:1 Hb:SNP solutions (SNP (3.24×10^{-6}mol dm^{-3}) itself did not show any significant absorption in this region of the visible spectrum). Again there was no significant change in comparing the spectra of the various Hb and Hb:SNP solutions, except with deoxyhaemoglobin (2.1×10^{-4}mol dm^{-3}) and SNP (0.05mol dm^{-3}) in excess. (SNP did not

Table 4

Solution	Absorption (nm)		
	Soret	α	β
methaemoglobin	405	500	
5:1 methaemoglobin:SNP	405	500	
oxyhaemoglobin	415	540	580
5:1 oxyhaemoglobin:SNP	415	540	580
deoxyhaemoglobin	430	558	
5:1 deoxyhaemoglobin:SNP	425	555	
deoxyhaemoglobin:SNP(excess)	420	540	570

interfere with this spectrum.) No other Hb:SNP sample, at these particular concentrations, exhibited any changes in the Hb spectrum

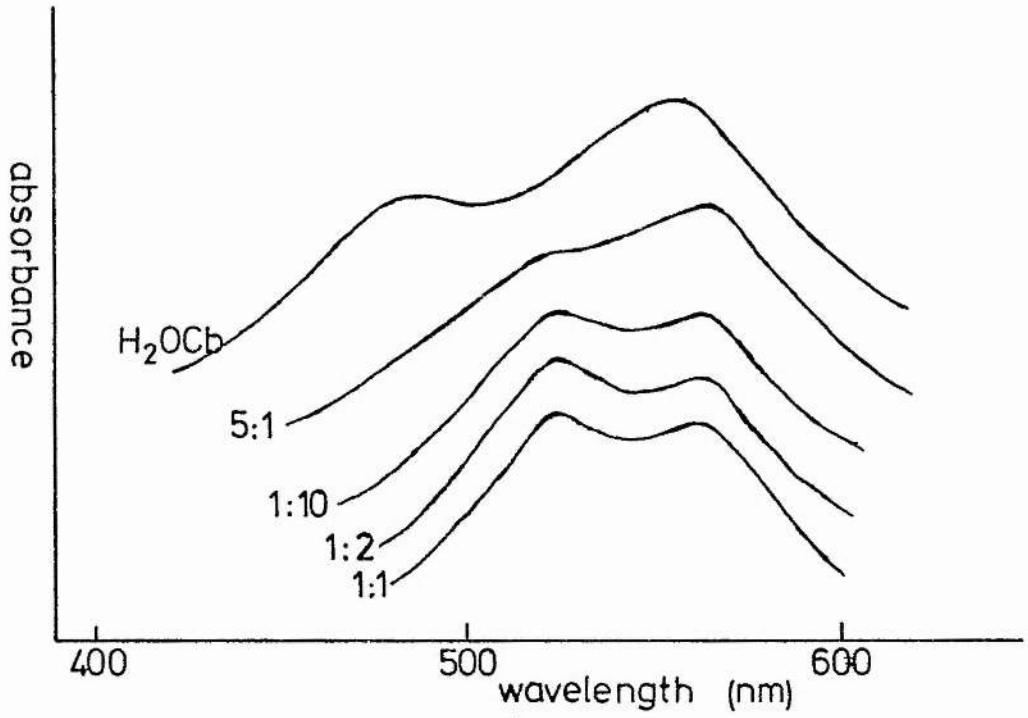
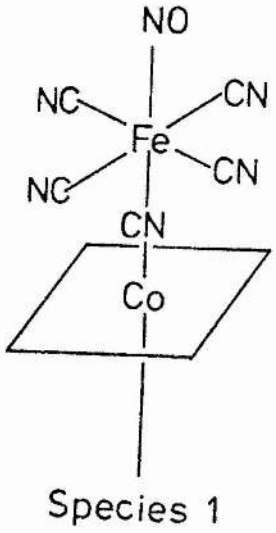
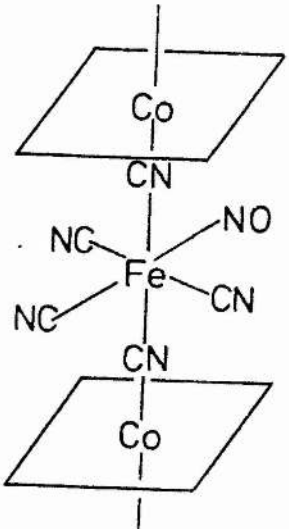


Figure 3 UV-Visible absorption spectrum of various mole ratios of H_2OCb:Nitroprusside.



Species 1



Species 2

(not shown in Table 4). The resulting spectrum looked similar to that of nitrosyl-haemoglobin (Soret 418, α 545, and β 575nm). At no stage in these uv-visible studies was there evidence of the production of metcyanohaemoglobin (Soret 415, α 530nm). This is in direct contrast to Smith and Krusyna[30] who claim SNP reacts with haemoglobin to give metcyanohaemoglobin and four free cyanides. This was supposed to occur by donation of an electron from oxyhaemoglobin to nitroprusside, so causing the nitroprusside to become unstable. This loss of an electron by O_2Hb causes the formation of metHb which would then react with one cyanide of the unstable nitroprusside to form metcyanohaemoglobin.

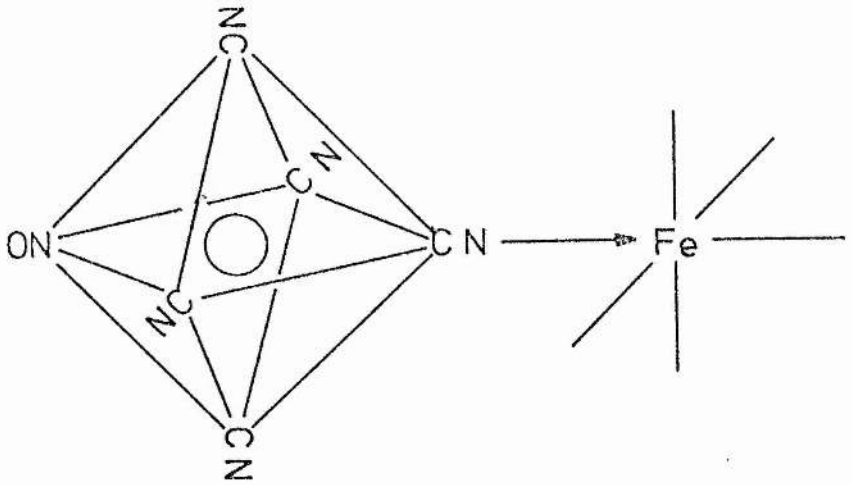
Since the interaction of SNP and aquocobalamin has been well documented by various different analytical techniques in previous chapters, the interaction of Hb and nitroprusside was studied at various significant molar ratios by uv-visible spectroscopy. This study should determine whether different binding species varied in their position in the visible spectrum, or merely their spectral shapes; therefore, allowing the interpretation of the various changes in shape and position of λ_{max} when SNP interacts with deoxyhaemoglobin at various ratios. At molar ratios of 1:1, 1:2, and 1:10 H_2OCb:SNP (as previously discussed) the predominant species in solution is Species 1, and at the molar ratio of 5:1 H_2OCb:SNP Species 2 will predominate (see Figure 3). Therefore, there are significant changes in the shape of the α and β bands at 532 and 512nm respectively due to the two different species, but no change in the position of the bands. Thus binding only causes changes in the shape of the uv-visible spectrum. In the case of deoxyhaemoglobin and SNP there must not only be

possible binding, but a chemical reaction to cause a shift in the position of the absorption.

^{13}C and ^{15}N NMR Studies of the Interaction of SNP and Haemoglobin

When the interaction of ^{13}C-labelled SNP (0.05mol dm^{-3}) and O_2Hb and MetHb (2.1×10^{-4}mol dm^{-3}) was studied by 20MHz ^{13}C NMR spectroscopy the resulting spectra were identical to the SNP second order spectrum (as discussed in Chapter 3). Even when the molar ratio of SNP and haem subunits was changed to 1:1 the SNP spectrum remained unperturbed. When a solution of SNP-^{13}C (0.05mol dm^{-3}) and deoxyhaemoglobin (2.1×10^{-4}mol dm^{-3}) in isotonic buffer pH 7.2 was recorded on a 90MHz ^{13}C NMR spectrometer a new AX_4 spectrum was observed. This spectrum showed δ_A = 139.7, δ_X = 138.9, and J = 18.7Hz, and a sharp singlet at δ = 177.9ppm as well as of the characteristic AX_4 spectrum of free $[Fe(^{13}CN)_5NO]^{2-}$ (see Chapter 3, p. 39). This sharp singlet resonance at 177.9ppm is characteristic of $[Fe^{13}CN)_6]^{4-}$ (see Chapter 3, p. 59). This new AX_4 species is indicative of an intact cyanoferrate species similar to the 1:1 SNP:H_2OCb complex (Species 1) discussed previously (p. 108). From the ^{13}C chemical shift of the new AX_4 spectrum it is apparent that the nitrosyl ligand is still present and by analogy to the 1:1 SNP:H_2OCb complex, Species 3 is formed by the complexation of the axial cyano ligand of $[Fe(^{13}CN)_5NO]^{2-}$ to the Fe(II) of the deoxyhaemoglobin.

Species 3



To determine whether the hexacyanoferrate(II) formed in the reaction did not further react with deoxyhaemoglobin the ^{13}C NMR spectrum of 90% ^{13}C-labelled $[Fe(CN)_6]^{4-}$ (0.05mol dm^{-3}) and deoxyhaemoglobin (2.1×10^{-4}mol dm^{-3}) was then recorded. The singlet resonance of hexacyanoferrate(II) was unperturbed by the presence of deoxyhaemoglobin.

It has been established that SNP reacts with thiols and sulphides to form coloured adducts.[31,32] When deoxyhaemoglobin is prepared by the method noted in the experimental section (p. 92), methaemoglobin is treated with a minimum quantity of reducing agent, sodium dithionite. Therefore, a control experiment was carried out to determine any interference by sodium dithionite in the SNP:deoxyhaemoglobin system. A ^{13}C NMR spectrum was recorded of a solution of 90% ^{13}C-labelled SNP (0.05mol dm^{-3}) to which was added a few drops of a

solution of sodium dithionite (0.1mol dm^{-3}). The spectrum obtained was a normal unperturbed second order spectrum of SNP. When the ^{13}C NMR spectrum of ^{13}C-labelled SNP (0.02mol dm^{-3}) and excess sodium dithionite (0.2mol dm^{-3}) was recorded a very broad baseline was observed after 6000 scans indicating the presence of a paramagnetic species, there were no diamagnetic species present. Therefore, these control experiments indicate that in the absence of deoxyhaemoglobin no $[Fe(^{13}CN)_6]^{4-}$ is formed from $[Fe(^{13}CN)_5NO]^{2-}$, and once the hexacyanoferrate(II) is formed it does not react further with deoxyhaemoglobin.

From these ^{13}C NMR studies no 2:1 complex of Hb:SNP, analogous to H_2OCb:SNP, was observed. This seems reasonable if the steric interactions involved are considered.

In comparing the two interactions, SNP:haemoglobin and SNP:cobalamin it is evident that the major difference between the two systems is that of ligand reorganisation in the SNP:haemoglobin system with the formation of hexacyanoferrate(II). Although $[Fe(CN)_6]^{4-}$ binds readily to the cobalt(III) of cobalamin (see Chapter 3) it shows no evidence of interaction with the Fe(II) of haemoglobin. In the reaction of SNP-^{13}C and deoxyhaemoglobin, as monitored by ^{13}C NMR spectroscopy, apart from unbound $[Fe(^{13}CN)_6]^{4-}$ and bound $[Fe(^{13}CN)_5NO]^{2-}$ no other ^{13}C-labelled diamagnetic species was detected. In particular neither free $(^{13}CN)^-$ nor $H^{13}CN$ were detected in any of the spectra. This <u>in vitro</u> work refutes the claims made by previous workers[30,33] to have detected release of free cyanide in the reaction of SNP and haemoglobin <u>in vitro</u>.

It has been reported by DeRubertis et al[34] that to activate guanylate cyclase by the addition of nitroprusside both a protein-bound haem and a reducing agent, e.g. dithiothreitol, cysteine, etc, are required to produce NO-haemoglobin. This is contrary to previous reports (p. 106). DeRubertis et al reported that if deoxyhaemoglobin and SNP are incubated together only 10% conversion of deoxyhaemoglobin to NO-haemoglobin occurred. However, Antman et al[35] found by infusing N-acetyl-L-cysteine with a vasodilator, nitroglycerin, that there was an increased activation of guanylate cyclase in vivo. With these two independent reports in mind a ^{13}C NMR spectrum was recorded of SNP (5.7×10^{-3}mol dm^{-3}) and deoxyhaemoglobin (5.7×10^{-2}mol dm^{-3}) in the presence of NAC (5.7×10^{-2}mol dm^{-3}) at pH 7.6 to determine any change in the diamagnetic species produced. This resulted in exactly the same species as found in the reaction of SNP and deoxyhaemoglobin.

In an attempt to further analyse the reaction of SNP and deoxyhaemoglobin in the presence and absence of N-acetyl-L-cysteine, a series of ^{15}N NMR spectra were run to monitor the nitrosyl of the SNP in the reaction. Initially, the spectrum of $[Fe(CN)_5NO]^{2-}$ (0.1mol dm^{-3}) and NAC (0.1mol dm^{-3}) gave only one ^{15}N resonance of δ = -10.1ppm attributed to $[Fe(CN)_5{}^{15}NO]^{2-}$. Alternatively, when the same spectrum was run using ^{15}N-labelled SNP (5.75×10^{-2}mol dm^{-3}) in the presence of NAC (5.75×10^{-2}mol dm^{-3}) and in the presence of the haem moiety (5.75×10^{-2}mol dm^{-3}) a new ^{15}N resonance appeared at δ = +13.44ppm, instead of at δ = -10.1ppm. This new resonance also appeared in the spectrum of $[Fe(CN)_5{}^{15}NO]^{2-}$ and haem moiety at the

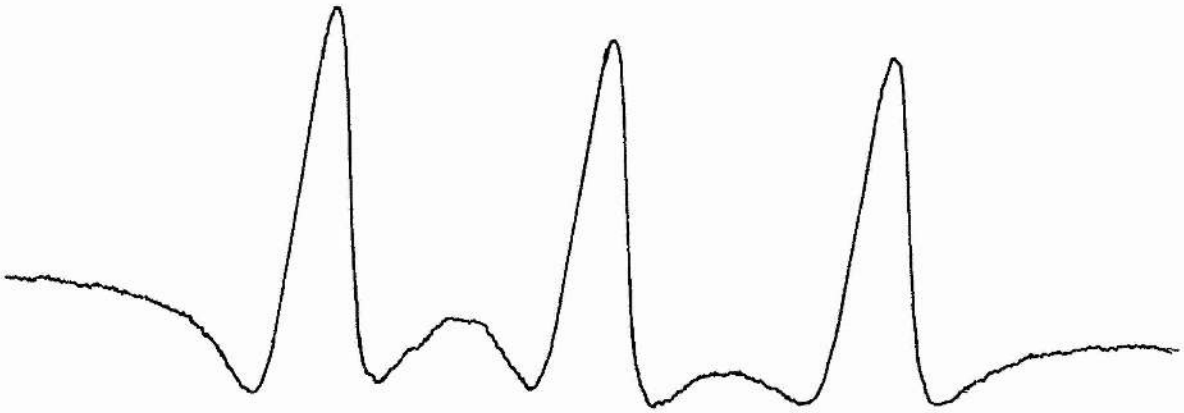


Figure 4 EPR spectrum (degassed) of mononitrosyl species $[Fe(CN)_5NO]^{3-}$, ^{15}N = 15.2, g = 2.028

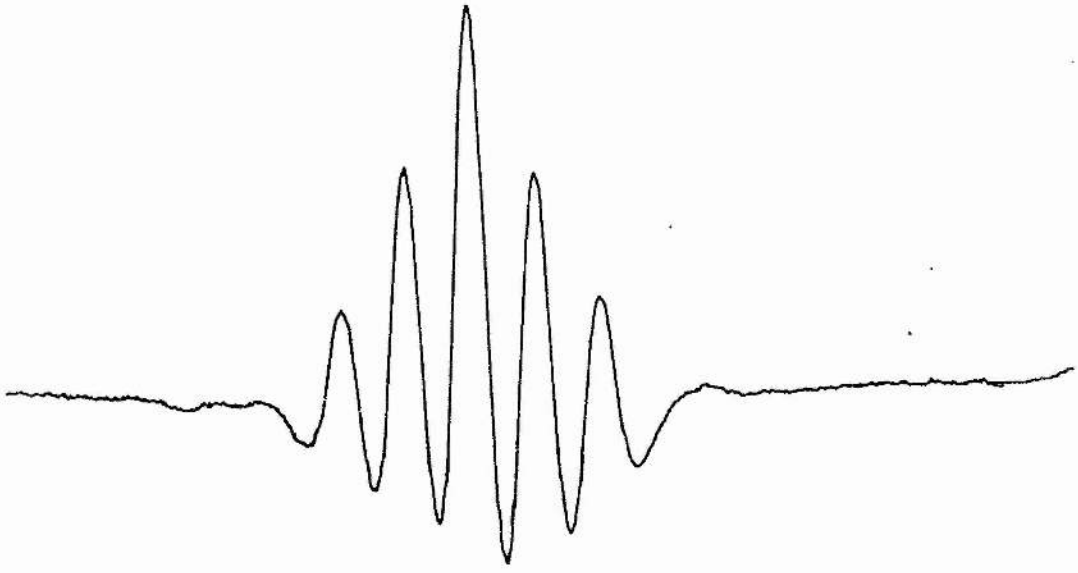


Figure 5 EPR spectrum (non degassed) of $[Fe(CN)_5NOH]^{3-}$.

same concentrations a previous ^{15}N spectra. This resonance is still characteristic of a trivalent nitrogen bound to oxygen, e.g. NO bound to the $[Fe(CN)_5]^{3-}$ moiety of SNP. This diamagnetic species is the 1:1 SNP:Hb complex. Therefore, there is no need for the presence of a reducing agent to produce the diamagnetic species of the reaction of SNP and deoxyhaemoglobin.

EPR Spectroscopy Studies

From both ^{13}C and ^{15}N NMR spectroscopy the diamagnetic species produced have been successfully identified. EPR spectroscopy studies were undertaken to identify any paramagnetic complexes produced in this reaction. The formation of paramagnetic nitrosyl-haemoglobin from nitroprusside and deoxyhaemoglobin is well established.[36] In this study the paramagnetic species was identified by uv-visible spectroscopy, since EPR identification involves solid state studies. However, from the reaction of deoxyhaemoglobin and $[Fe(CN)_5NO]^{2-}$ an additional persistant paramagnetic mononitrosyl complex (see Figure 4) has been observed. This complex is characterised by $g = 2.028$ and $A(^{15}N) = 15.2$; the same species was also obtained from the reaction of SNP and haem moiety. Mulvey and Waters[32] reported that the same complex was formed by the electrochemical reduction of $[Fe(CN)_5NO]^{2-}$, or by chemical reduction using $NaBH_4$, $Na_2S_2O_4$, or ascorbic acid, and they identified the mononitrosyl species as $[Fe(CN)_5NO]^{3-}$. Using EPR spectroscopy an identical complex was observed with deoxyhaemoglobin and haem (both at pH 7.2), and L-cysteine, NAC and glutathione (all at pH 7.6), as well as with $Na_2S_2O_4$ as reductants for $[Fe(CN)_5NO]^{2-}$ in degassed systems. In non-degassed the spectrum observed in the

reaction of $Na_2S_2O_4$ and SNP was that characteristic[32] of $[Fe(CN)_5NOH]^{3-}$ (see Figure 5) formed by further reduction of $[Fe(CN)_5NO]^{3-}$ to $[Fe(CN)_5(H_2NOH)]^{3-}$ followed by aerial oxidation. When $[Fe(CN)_5NO]^{2-}$ was partially reduced by $Na_2S_2O_4$ this yielded solutions containing both $[Fe(CN)_5NO]^{3-}$ (characterised by EPR spectroscopy), and unchanged $[Fe(CN)_5NO]^{2-}$ the ^{13}C NMR spectrum of which was unperturbed (see p. 110). These observations illustrate the independence of the ^{13}C NMR spectra of diamagnetic cyanoferrate complexes from the paramagnetic co-solutes. On the spectroscopic time scale the electron exchange between $[Fe(CN)_5NO]^{2-}$ and $[Fe(CN)_5NO]^{3-}$ must be slow.

Previous workers in this department[37], using uv-visible spectroscopy observed also the formation of methaemoglobin (Fe(III)) in the reaction between $[Fe(CN)_5NO]^{2-}$ and deoxyhaemoglobin. This observation helps to complete the overall conclusions that can be made from this reaction. Figure 6 is a scheme summarising the products from the interaction of $[Fe(CN)_5NO]^{2-}$ with deoxyhaemoglobin, and accounts for all of the reaction products identified by EPR, ^{13}C NMR, and uv-visible spectroscopy. This scheme also accommodates, by means of a ligand redistribution step coverting the intermediate $[Fe(CN)_5]^{3-}$ (III) into the observed $[Fe(CN)_6]^{4-}$ (V), the complete absence of any free cyanide found in the present work using isolated haemoglobin, or with whole blood.[38] The mechanistic key to the very rapid hypotensive activity of $[Fe(CN)_5NO]^{2-}$ may be provided by the ready formation of $[Fe(CN)_5NO]^{3-}$ through the reduction of $[Fe(CN)_5NO]^{2-}$ by a wide range of molecular species present in normal mammalian biochemistry, demonstrated in this work.

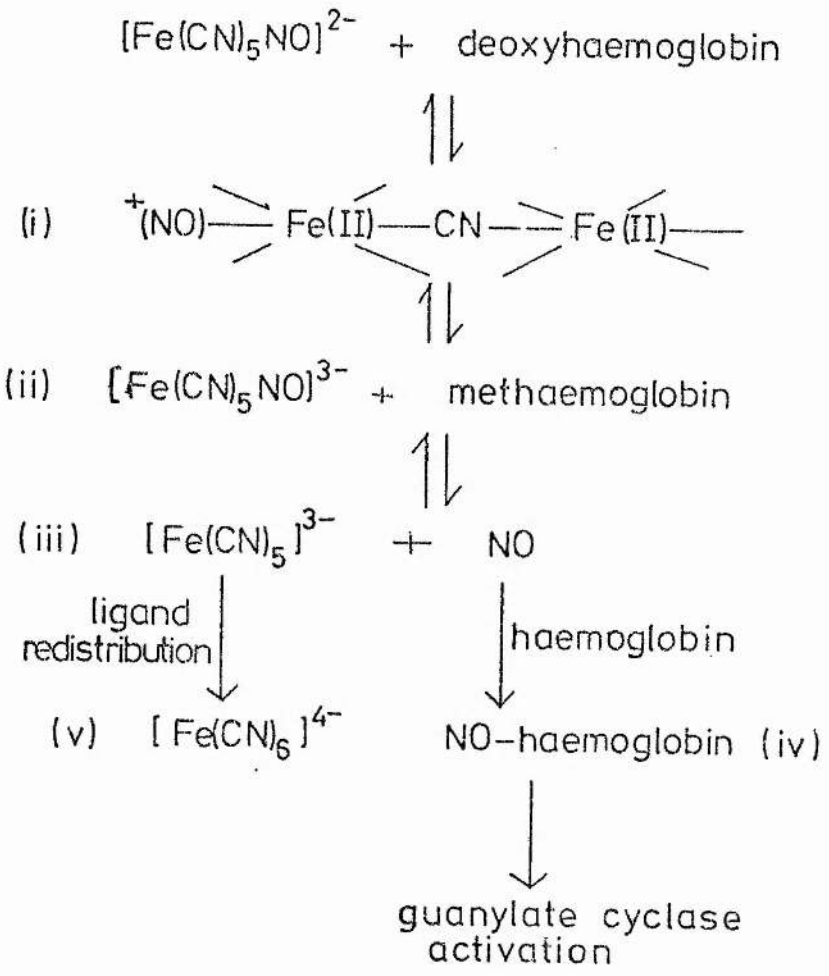


Figure 6 Scheme showing the in vitro reaction of SNP and deoxyhaemoglobin.

Since both (I) and (II) are formed using only deoxyhaemoglobin but not oxyhaemoglobin, this indicates that (I) is the primary intermediate in the formation of (II) and production of NO-haemoglobin (IV). This conversion of (I) to (II) requires an inner sphere electron transfer across the Fe-C-N-Fe bridge. This study has successfully observed the bridged intermediates (I) and the two products, $[Fe(CN)_5NO]^{3-}$ (II) and metHb, of the deoxyhaemoglobin and nitroprusside redox reaction. In the redox reaction the π^* orbital of

the NO in $[Fe(II)(CN)_5NO]^{2-}$ being the LUMO will receive the electron, therefore reducing the nitrosyl ligand from NO^+ to $NO^\cdot$ while still maintaining the iron of $[Fe(CN)_5NO]^{3-}$ as Fe(II). Similarly when SNP reacts with thiols $[Fe(CN)_5NO]^{3-}$ (II) is formed. The thiol (RS^-) acts as a nucleophile[20,31,39] by addition at the nitrosyl ligand to produce an intermediate coloured adduct $[Fe(CN)_5N(O)SR]^{3-}$, which then loses SR.

In spite of the considerable amount of work[40-42] carried out on the mechanism of guanylate cyclase activation by SNP, and other nitrosyl containing species, the action still remains unknown. In the past conclusions were drawn from studies with crude preparations of guanylate cyclase, or with intact cells which gave misleading and incorrect results. More recently significant progress has been made by greater purification of guanylate cyclase.[36,43] As a result the two schools of thought discussed previously have arisen. From this present study of the reaction of deoxyhaemoglobin and SNP, and the ready formation of $[Fe(CN)_5NO]^{3-}$; the precursor of nitrosyl-haemoglobin which is a potent activator of purified guanylate cyclase[19] illustrates a ready path of activation of guanylate cyclase by SNP. Although this chemical study has been carried out <u>in vitro</u> with isolated haemoglobin in the case of guanylate cyclase activation, this study may be of great biological significance since recent work[44] in this department has shown that SNP can readily transfer across the red cell membrane and hence interact with haemoglobin. More importantly the haem moiety is thought to be an integral part of the soluble bovine guanylate protein[45] cyclase unit and is important in the activation of this enzyme.

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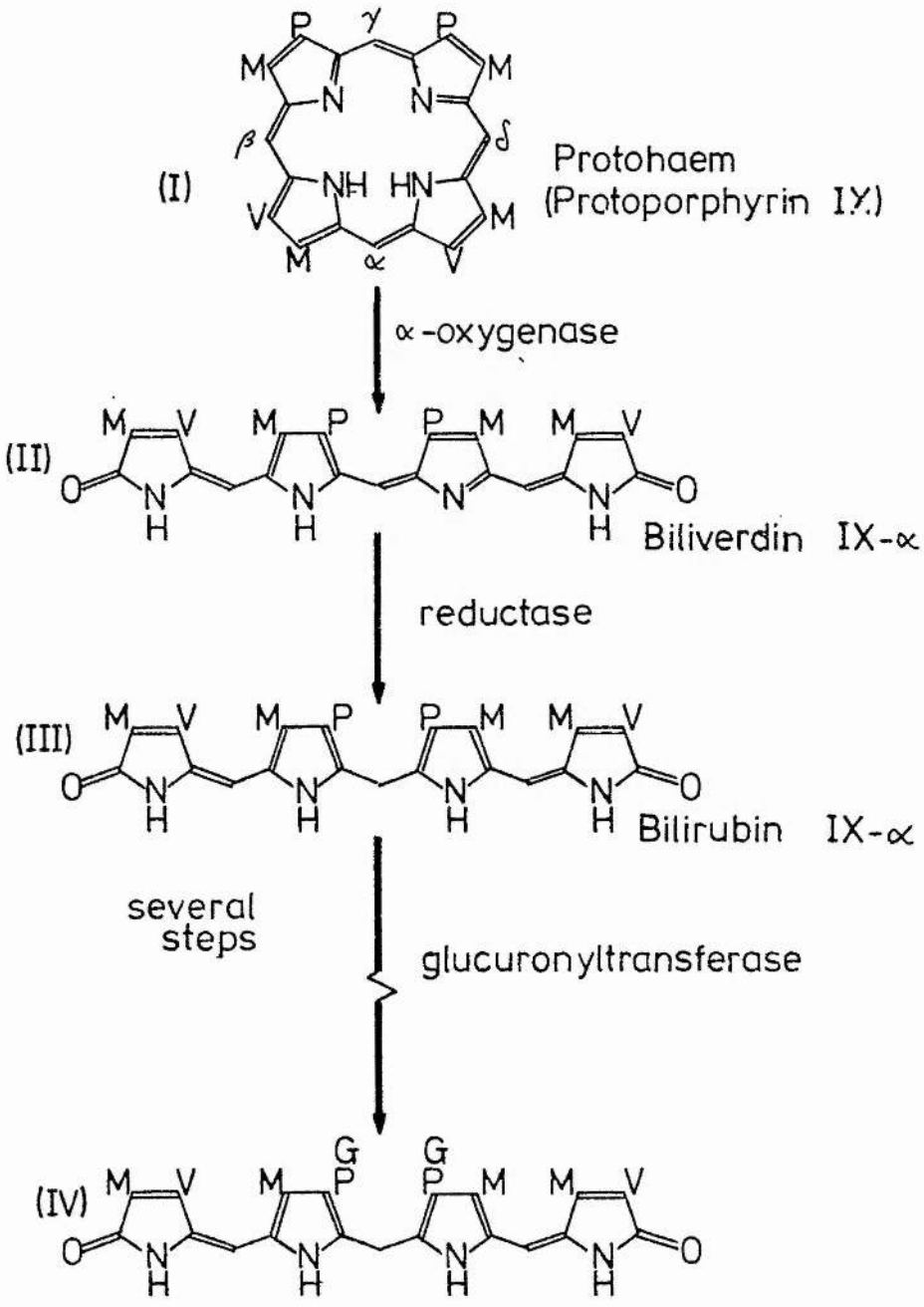
Part 2

Chapter 5

REVIEW OF BILIRUBIN ASSAY


(I)
Protohaem
(Protoporphyrin IX)
α-oxygenase
(II)
Biliverdin IX-α
reductase
(III)
Bilirubin IX-α
several steps
glucuronyltransferase
(IV)
M = CH3 V = CH=CH2
P = CH2CH2COOH
PG = CH2CH2COOG
G = Glucuronic acid

Scheme 1

5.1 INTRODUCTION

Bilirubin IXα (III), a tetrapyrrole, (Photograph 20) is found at low concentrations of 15 μM in normal adult human serum. It originates primarily from the catabolism of the haem moiety of haemoglobin[1] (Photograph 19). The nature of the reaction and mechanism of haem degradation has received considerable attention over the years[2-5] from both a physiological and chemical viewpoint. There is a substantial body of evidence to suggest haem catabolism occurs by essentially the same mechanism as haem degradation in chemical model systems. In mammalian systems all bilirubin produced is derived from the degradation of protohaem (I) (see Scheme 1) and the intermediate tetrapyrrolic precursor of bilirubin is biliverdin (II) ; a blue green pigment. The enzyme responsible for this conversion is biliverdin reductase (EC 1.3.1.24).[6] Other enzymes involved in the haem catabolism are microsomal haem decyclising oxygenase (EC 1.14.99.3)[7] and NADPH-cytochrome a_2 reductase (EC 1.6.2.5).[8] The sites of haem breakdown and bilirubin production are predominantly the spleen and liver, and to a lesser extent in the blood stream.[2,4]

The bilirubin IXα acid formed from the breakdown of haemoglobin has remarkable intramolecular hydrogen bonding.[9,10] As a result this yellow pigment is insoluble in water and has to be transported in the plasma bound to protein. Serum albumin is the major carrier[11,12], but small amounts of bilirubin have been identified with other serum proteins such as the α and β globulins.[13,14] Jacobsen [15] found that bilirubin is bound to human albumin at a single high affinity site and obtained evidence for two weaker sites with affinities 10 to

100 fold lower. The high affinity site is thought to be a hydrophobic pocket where binding takes place between the primary amino group of the protein and the carboxylic group of the bilirubin. In particular, Jacobsen[16] reports lysine residue 240 of human serum albumin is involved in the high affinity binding of bilirubin. Further aspects of bilirubin binding are investigated in Chapter 6 of this present work.

Albumin transports the bilirubin to the liver[17] for processing and excretion, where efficient elimination of bilirubin is accomplished by conjugation with polar substances such as glucuronic acid. This enzymatic esterification process is carried out by a microsomal enzyme, UDP-glucuronate (glucuronyl transferase (EC 2.4.1.17)) with uridine diphosphate acid (UDPGA) as the glucuronyl donor.[18,19] The bilirubin ester of glucuronic acid exists in the serum exclusively as diglucuronide. Although no monoglucuronide has been found in serum there is evidence[20] to support the view that bilirubin is glucuronidated in two steps, and that monoglucuronide is an intermediate. The bilirubin is now water soluble, non toxic, and is excreted through the liver cells in the bile capillaries. Following excretion through the bile into the intestines the glucuronide is cleaved by bacterial action and reduced further before excretion.

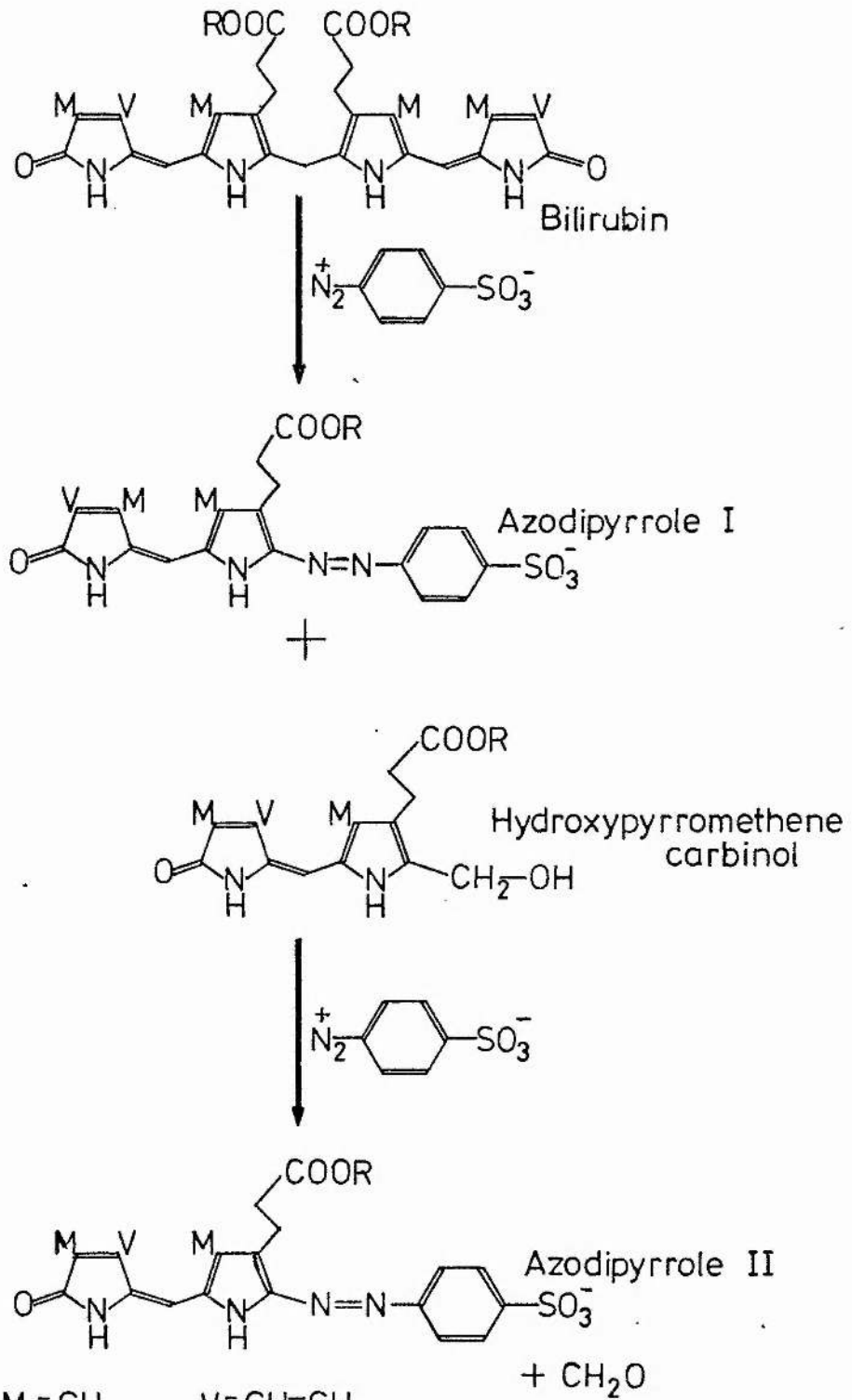
Up to about 300mg of bilirubin per day is produced as a degradation product of haem through the degradation of mature, aged erythrocytes in the reticuloendothelial system. Those suffering from conditions such as liver disorders, pernicious anaemia and any disease which causes haemoglobin breakdown or disrupts one or more of the steps between production and excretion of bilirubin[21-23] develop

hyperbilirubinaemia ; elevated bilirubin concentrations in serum. Berthelot *et al*[24] have dealt with in some detail the various causes of hyperbilirubinaemia, but a lot of attention has been given to research into the human neonates underdeveloped capability of conjugation and excretion.[25] This results in yellow staining of the skin, i.e. jaundice, normally increasing until the 4th or 5th day of life. When excretion starts the jaundice subsides. In premature babies hyperbilirubinaemia can be more acute due to delay in development of excretory functions, or when the infant receives a drug which is bound to albumin and occupies the binding site of bilirubin. Under conditions of excess free bilirubin the molecule binds to the enzyme carrying membrane in the cell and impedes cell functions. In new born babies irreversible cell damage can occur, especially lasting brain damage; kernicterus, due to the passage of bilirubin through the blood-brain barrier.

5.2 THE PROBLEMS OF BILIRUBIN ESTIMATION IN HUMAN SERUM ALBUMIN

The van den Bergh Test

In recent years much new knowledge has been amassed on the structure and chemistry of bilirubin. This has been coupled with a wide range of spectroscopic[26], chromatographic[27], chemical[28], and enzymatic[29] techniques to estimate bilirubin concentrations in human serum. However, the most commonly used clinical technique is a modification of a method devised at the end of the nineteenth cen-



M = CH_3 V = $CH=CH_2$
R = H, unconjugated bilirubin
R = Glucuronic acid, conjugated bilirubin

Scheme 2

tury. Ehrlich[30] discovered that bilirubin reacts with p-diazobenzene sulphonic acid in the presence of alcohol to form azopigment which is blue in both acidic and alkaline pH, and red near neutral pH. These intense colours are due to the production of oxydipyrromethane azo compounds (I) and (II) (see Scheme 2).[31] The reaction proceeds in two steps, each giving rise to equal amounts of azo pigment, the central methylene bridge being released as formaldehyde[32] in the presence of excess arenediazonium ion. Using this chemical test Ehrlich[30] could successfully detect bilirubin in urine. More importantly in 1943 van den Bergh and Snapper[33] applied this diazo reaction to serum, which is clinically more useful. Before the serum test could be carried out all the serum protein had to be removed which was thought to inhibit the diazo reaction, the addition of alcohol caused the proteins to precipitate out. The test for bilirubin was carried out under mildly acidic conditions necessary for diazotisation of sulphonilic acid and gave rise to the red azo pigment. Later van den Bergh and Muller[34] observed two azo reactions upon addition of his diazo reagent (0.3l of 0.5% sodium nitrite and 10ml of acidified 0.1% sulphonilic acid) to serum. The first was the "direct" reaction in which the colour develops within 30 seconds in the absence of alcohol. The second reaction, or "indirect" reaction, was observed when protein in the serum was precipitated by ethanol. The resulting supernatant was used and the colour then developed. Therefore, by the addition of alcohol van den Bergh measured the total bilirubin content of serum, i.e. the sum of the "direct" and "indirect" reactions. After many years of research several reasons[35] have been put forward to explain the difference between "direct" and "indirect" forms of bilirubin in the van den Bergh test. Now it is generally accepted that the "direct" reaction is due to the reaction of conjugated bili-

rubin, i.e. bilirubin diglucuronide, with reagent along with a small quantity of unesterified bilirubin. In the "indirect" reaction van den Bergh had solubilised the unconjugated bilirubin so that it could react with diazo reagent. Therefore, "indirect" consists solely of unesterified bilirubin. This method was a forerunner of quantitative methods[35], some of which are still in use to this date. However, all of these techniques suffer from a major disadvantage in that some of the bilirubin co-precipitates with the protein. This leads to an underestimation of the total bilirubin.

Malloy and Evelyn[36] introduced a technique in which ethanol, or methanol is used to solubilise unconjugated bilirubin, but not precipitate proteins. The conjugated and unconjugated bilirubin can be measured by estimation before and after the limited alcohol addition. The major drawback of the many modifications to this method is the fact that samples require high dilutions prior to assay. Thus little colour is developed for normal samples and there are problems of turbidity.[37,38]

Other modifications of the van den Bergh test have been developed using "accelerators" or "promoters" avoiding the need for using alcohol. There are several substances which accelerate the azo coupling of unesterified bilirubin in aqueous solution, these include caffeine, sodium benzoate, sodium acetate, urea, and mixtures of these.[39] Jendrassik and Grof[40] used a caffeine-sodium benzoate mixture to promote azobilirubin formation reading the azobilirubin at alkaline pH. This method is used routinely to measure total bilirubin concentration in the plasma. It is claimed that these "accelerator"

methods increase both the rate of reaction and colour intensity[39] in the "indirect" bilirubin. It is suggested that these "accelerators" alter the bilirubin-protein complex in such a way that makes the bilirubin more open to attack by the arenediazonium ion. In the present study Chapter 6 details fluorescence studies undertaken to estimate the interference of "accelerators" with the bilirubin-albumin complex.

In summary the need to quantify the van den Bergh test has led to the many modifications developed today.[36,40] As a result, there are many procedures, various combinations of arenediazonium salts, pH reading times, solvents, and "promoters". They all claim to be quantitative, but no two give the same results particularly in the "direct" bilirubin readings. Many factors lead to conflicting results, i.e. the time allowed for colour development is crucial. The same diazo method can yield very different results if the readings are not taken consistently at the same time.[41] In addition haemoglobin[42,43] causes interference with the diazo methods, resulting in low values. These low results may be due to :-

(i) oxyhaemoglobin oxidising bilirubin,

(ii) increased absorbance of a sample blank due to haemoglobin,

(iii) increased destruction of the azobilirubin with haemoglobin.

Nevertheless, the diazo method still plays a major role in the routine clinical determination of bilirubin. In recent years the demand for a rapid and convenient spot test for bilirubin lead Eastman-Kodak to develop a diazo based dry film[44,45] for the determination of total bilirubin in serum. In Chapter 7 this is dis-

cussed in more detail, and the development of an optimum azo compound to incorporate into these films was undertaken.

Alternative Methods of Estimating Bilirubin Content in Human Serum

Apart from chemical estimation of bilirubin, the other most commonly used is the direct spectrophotometric method.[26] This depends upon dilution of the sample with buffer, or saline, and reading the bilirubin absorbance peak at 460nm and again at 522nm to correct for haemolysis. This method is useful for pediatric cases with hyperbilirubinaemia when non-bilirubin absorbing substances such as carotenes are diluted out to insignificant proportions when compared to bilirubin. This method measures total bilirubin and allows a total estimation to be performed on small samples of blood. This technique tend to give superficially high results for bilirubin since it is an unspecific method for measuring bilirubin content.

High Performance Liquid Chromatography[27] can also estimate concentrations of bilirubin in serum. By using ion-pair reverse phase HPLC, the conjugated and unconjugated bilirubin in serum may be assayed without the need to derivatise, but the analysis is still very lengthy.[46]

There are two methods in current use for the measurement of unbound bilirubin; Sephadex G-25 chromatography[47] and enzymatic determination with peroxidase.[48] Sephadex retains unbound bilirubin on the column, whereas albumin bound bilirubin is eluted. The unbound

bilirubin is detected by a diazo reaction *in situ*[49], or eluted and quantitated by spectrophotometry.[50] This technique overestimates the amount of unbound bilirubin.[51] Samples containing conjugated bilirubin give false positive test for the presence of unbound bilirubin.[52] The column technique interferes with the equilibrium of the albumin bound and unbound bilirubin.

Horseradish peroxidase catalyses bilirubin oxidation with hydrogen peroxide. The peroxidase will react with the unbound bilirubin only.[29] The bilirubin peroxide reaction can be accelerated by drugs.[53] This technique tends to give low concentrations of unbound bilirubin.[54] Another enzymatic technique employs bilirubin oxidase. This enzyme oxidises bilirubin to biliverdin, therefore decreasing bilirubins specific absorbance at 440-460nm. The biliverdin is also oxidised, but at a much slower rate of 2%. The oxidase will only react with conjugated bilirubin, but will also react with unconjugated bilirubin if solubilised with salycylic acid or sodium cholate. The method is not very sensitive and requires a 30 minute incubation time to convert bilirubin to biliverdin, which is not convenient.

As a consequence of the drawbacks of these alternative methods of bilirubin assay, the diazo method is still more commonly used. It still gives the least inaccurate results and in comparison to some is a much more rapid means of bilirubin determination. However, as previously discussed, there is much room for improvement in the diazo method.[55]

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Chapter . 6

FLUORESCENCE AND SOLID STATE STUDIES OF

BILIRUBIN TO ALBUMIN BINDING

Abbreviations

HSA = Human Serum Albumin

BSA = Bovine Serum Albumin

SDS = Sodium Dodecyl Sulphate

CPMAS = Cross Polarisation Magic Angle Spinning

TOSS = TOtal Side band Suppression

6.1 INTRODUCTION

The unconjugated bilirubin IXα -z,z formed from the breakdown of haemoglobin[1] shows extremely low solubility in water; it is calculated to be in the order of 10^{-9} mol dm^{-3}.[2] This extremely low solubility is attributed to the extensive intramolecular hydrogen bonding of bilirubin (see Figure 1).

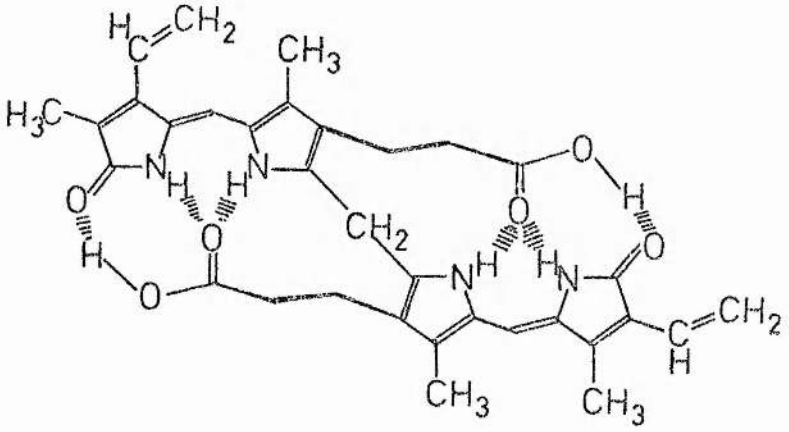


Figure 1 Intramolecular hydrogen bonding of Bilirubin.

At pH 7.2 the hydrogen bonding is so extensive that all the bonding moieties of the molecule are saturated, leaving the hydrophobic groups at the outer aspect of the molecule. As a result of its aqueous insolubility, near physiological pH, non conjugated bilirubin acid tends to form aggregates.[3] At pH 7.4-8.0 Brodersen[4] has observed colloid behaviour of bilirubin acid. The neurotoxicity of bilirubin (kerniterus) can therefore be adequately explained by this behaviour. The intramolecular hydrogen bonding renders the molecule insoluble in the plasma. The lipophilic moieties become exposed and aggregates of the bilirubin dianions form in the phospholipid mem-

branes of the cells disrupting the membranes structure and hampering the activity of the attached enzymes. In normal conditions this lipophilic behaviour leading to neurotoxicity is never observed since all the bilirubin dianions circulate in the blood tightly bound to serum albumin.

In spite of the fact that serum albumin has been one of the most thoroughly studied proteins, its three dimensional structure has not yet been determined. This protein contains only one peptide chain

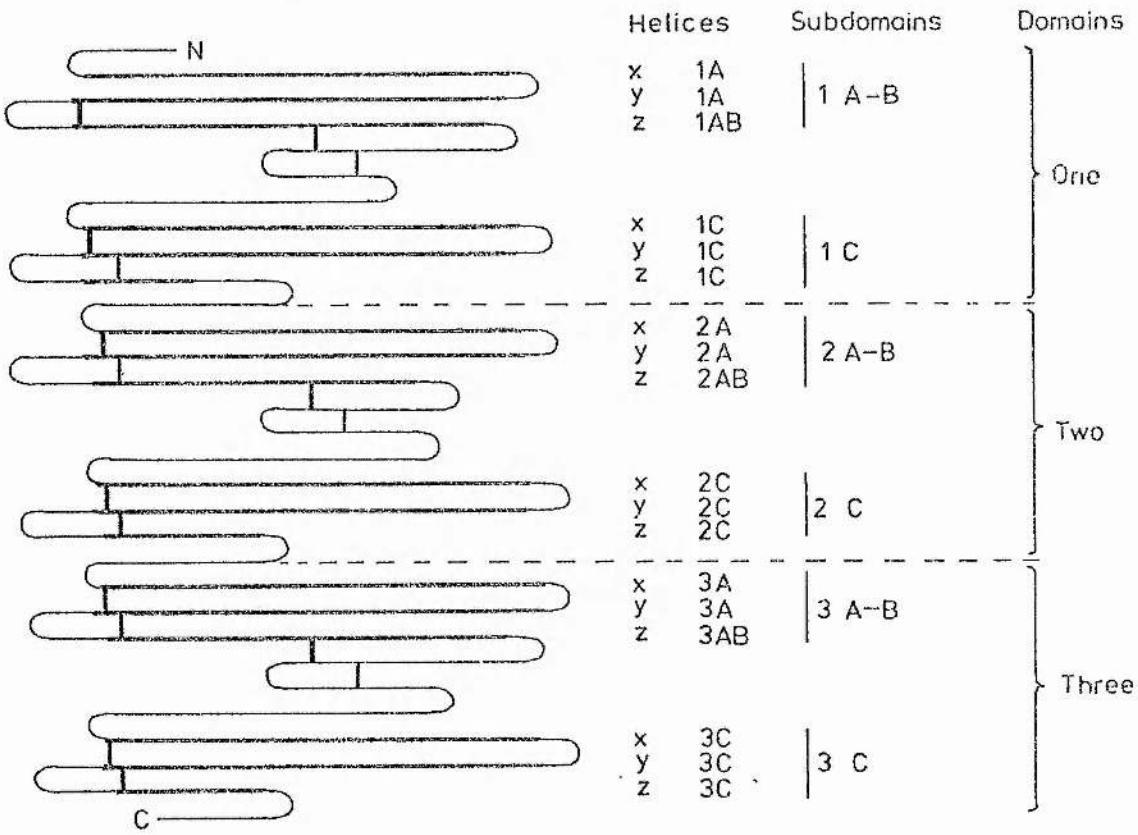


Figure 2 Secondary structure of Bovine Serum Albumin , adapted from the model of Brown.[5,6]

with some 580 amino acid residues (see Appendix 2 for full listing).

The chain is folded in a unique pattern of double loops.[7]

Recent models of albumin molecules (Figure 2) illustrate the proteins secondary structure as having three so-called domains; anion binding sites and six subdomains. Each subdomain contains three parallel α helices, x,y, and z, each having approximately 22 amino acid residues. This model can explain many of the binding characteristics. As discussed previously (Chapter 4, p. 104) albumin is a universal protein carrier, binding inorganic ions, organic anions, and uncharged species. In 1929 Bennhold[8] was the first to emphasize the biological importance of unconjugated binding to albumin[9] in understanding bilirubin transport and toxicity.

Jacobsen[10] found that bilirubin is bound to albumin at a single high affinity site and obtained evidence of two weaker sites with affinities 10 to 100 fold lower. Brodersen's[11] stoichiometric studies have found that three bilirubin dianions are internalised within a molecule of human serum albumin (HSA). Techniques of affinity labelling[12,13], chemical modification, and cleavage of proteins by enzymatic hydrolysis[14,15] all indicate that for both bovine and human serum albumin region 2A-B and a small portion of subdomain 1C (see Figure 2) is the high affinity binding site for bilirubin. This has the best composition for bilirubin binding.[5,6] The secondary binding site of the bilirubin dianion is between the half domains 3A-B.

The actual binding of the bilirubin dianion is fast, reversible, and accordingly proceeds to an equilibrium. Jacobsen[16,17] found that the binding takes place through several steps; a very fast combination of the two molecules followed by four consecutive conformational changes of the bilirubin:albumin complex. This may indicate that the bilirubin molecule contacts the albumin in one spot where upon the protein is modelled around the bilirubin molecule. The binding affinity is high and the equilibrium is independent of pH between 7 and 9 for HSA. The primary binding constant of bilirubin to HSA is $5.9 \times 10^{7} dm^{3} mol^{-1}$.[2] Details of binding of bilirubin to HSA at a molecular level are unknown, but thermodynamic calculations carried out by Jacobsen[18] suggested that the binding at the primary sites is driven by a strong enthalpy force. Kuenzle[19] proposed a structure for bilirubin binding to albumin involving hydrogen bonding. This however implies that bilirubin acid is bound and not the dianion.

In the high affinity binding site (residues 180-250) of bilirubin in human serum albumin is a tryptophan amino acid residue (214). This single tryptophan residue in human albumin is a chromophore which exhibits an intrinsic fluorescence.[20] This fluorescence can be utilised as a probe to study the binding of bilirubin dianions. Bovine serum albumin (BSA) has two tryptophan residues, both in the high affinity site for bilirubin. Therefore, BSA was employed in this present study to investigate bilirubin binding by the technique of fluorescence spectroscopy. In addition bilirubin exhibits an extrinsic fluorescence. Free in solution at pH 7.2 bilirubin exhibits

negligible fluorescence, however as it interacts with albumin its fluorescence increases. This property of extrinsic fluorescence coupled with tryptophan fluorescence was employed initially to establish the binding capacity and affinity of albumin for bilirubin under specific *in vitro* conditions.

In the diazo method of Jendrassik and Grof[21] (as discussed in Chapter 5, p. 125) "accelerators" are employed to promote the azo coupling of unesterified bilirubin of the "indirect" reaction. How these "accelerators" work remains a mystery, since they appear to have little in common chemically. It is suggested that they alter the bilirubin:protein complex in such a way as to make the bilirubin more open to attack by the arenediazonium ion. The present study employed the fluorescence quenching technique to monitor the effects of these "accelerators" and mixtures of them on the bilirubin:albumin complex.

Marr-Leisy *et al*[22] using the technique of Induced Circular Dichroism have recently reported that at pH 11.4 bilirubin binds to the α helical conformation of poly-L-lysine. This binding seems a very appropriate topic to study using the technique of fluorescence to measure any enhancement of bilirubin emission fluorescence due to addition of poly-L-lysine and poly-L-asparagine at pH 11.4 and pH 7.4 respectively. These two poly amino acid chains are of particular interest in elucidating the interaction of bilirubin with albumin. Lysine amino acid residue 240 in HSA and the corresponding lysine (residue 238) in BSA is involved in the high affinity binding of bilirubin.[12] Although the amino acid asparagine is not involved in the high affinity binding of bilirubin to albumin, the side chain is

polar and able to participate in hydrogen bonding. In the bilirubin:albumin complex hydrogen bonding is thought to play a major role, therefore studying any interaction will be informative. Details of such a study are presented in this work.

The second section of this chapter details the use of ^{13}C solid state NMR spectroscopy, initially to study bilirubin IXα-z,z, and a dipyrrylmethane in the solid state and compare these with solution state NMR. Then an attempt was made to study the high affinity binding site of bilirubin to bovine albumin by solid state NMR. Identical studies were carried out using solution state NMR but there were problems of solubility and hence are not reported in this work.

6.2 EXPERIMENTAL

6.2.1 FLUORESCENCE STUDIES

Materials and Methods

Bovine serum albumin (fatty acid and globulin free), bilirubin IXα-z,z, caffeine, urea, dyphylline, sodium dodecyl sulphate, poly-L-lysine, and poly-L-asparagine were all purchased from Sigma UK. Sodium benzoate and sodium acetate were purchased from BDH and were of AnalaR grade.

Bilirubin was purified by crystallisation from a chloroform-methanol mixture according to the procedure of Ostrow *et al.*[23] Purity was confirmed by thin layer chromatography. All other chemicals were used as received. Two buffered solvent systems were used throughout the fluorescence studies; isotonic buffer pH 7.2 (as prepared in Chapter 2, p. 18), and phosphate buffer pH 11.4.

Instruments

A Perkin-Elmer MPF-44A spectrofluorimeter was employed, which was linked to a NASCOH microcomputer. The spectra were displayed on an oscilloscope and the spectral data then transferred to a VAX 11/780 mainframe computer by the microcomputer and plotted.

Fluorescence Spectra

All spectra were recorded at ambient temperature (20-25°C) after typically accumulating 15 scans. For bovine serum albumin emission fluorescence solutions were activated by an excitation wavelength of 290nm and emission spectra recorded between 300 and 450nm. The emission maxima for the tryptophan residues was at 350nm. For bilirubin emission fluorescence solutions were activated at 438nm, and emission spectra recorded between 460 and 640nm. The emission maxima for bilirubin was recorded at 533nm.

Solutions for Fluorescence Studies

Stock Solutions (i) Bilirubin - these were prepared in 0.1mol dm^{-3} NaOH to ensure complete solubility, generally of concentration 1.6 x 10^{-5}mol dm^{-3}. These solutions were kept refrigerated and in the dark until used to prevent bilirubin photodegradation, this was monitored by visible spectroscopy (bilirubin λ_{max} 440nm). The solutions were generally used within six hours of preparation.
(ii) Bovine serum albumin - these were prepared in isotonic buffer pH 7.2 and kept cool and protected from light. A typical concentration of albumin stock solution was 1.5 x 10^{-5}mol dm^{-3}.

Solutions for Fluorescence Quenching Titrations : The titration consists of adding aliquots of quencher, bilirubin or other substrate, to bovine albumin solution (1.5 x 10^{-5}mol dm^{-3}) pH 7.2, and measuring the decrease in albumin fluorescence after each addition. Concentrations of quencher added varied depending on the exact stoichio-

metries required. In the case of bilirubin emission fluorescence quenching titrations, initial solutions contained a mole ratio of 0.73 bilirubin to albumin to ensure complete binding to which aliquots of bilirubin or "accelerators" were added and the decrease or increase in the bilirubin emission fluorescence was recorded after each aliquot addition. When measuring the effect of poly-L-lysine and poly-L-asparagine on bilirubin emission fluorescence, aliquots of the poly-amino acid were added to a phosphate buffer solution (pH 11.4 and pH 7.2) of bilirubin (1.05×10^{-5} mol dm^{-3}) and the fluorescence measured after each addition. In all fluorescence quenching titrations very small aliquots were used to prevent dilution effects on the fluorescences.

6.2.2 SOLID STATE NMR SPECTROSCOPY

All solid state ^{13}C NMR spectra were recorded on a Varian VXR-300MHz spectrometer with solid state attachment and an Oxford wide bore magnet at the S.E.R.C. Solid State NMR Service at the University of Durham. The carbon spectra were recorded at 75.43MHz. All chemical shifts were referenced to external TMS. The spectra were recorded at 25°C and obtained with a high power proton decoupler.

Solid State ^{13}C NMR of 3,3',4,4'-Tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane

This was prepared by the method of Fischer and Walach.[24] The dipyrrole solid state ^{13}C NMR was recorded at 25°C using the CPMAS mode with 150 transients and a delay of 500s between pulse widths of 8.0 s. The spectral width was 20000Hz. To remove spinning side bands the ^{13}C dipyrrole spectrum was then run in both CPMAS and TOSS modes with 300 transients and a delay of 5.0s with a pulse width of 8.0 μs.

Solid State ^{13}C NMR of Bilirubin

The sample of bilirubin was purified by the method discussed previously (p. 139). The ^{13}C NMR spectrum was recorded using CPMAS and TOSS modes with 128 transients and a delay of 5.0s with a pulse width of 8.0 μs. The spectral width was 30030Hz.

Solid State ^{13}C NMR of Bovine Serum Albumin

Fatty acid and globulin free bovine serum albumin was shown by TLC to be pure. The ^{13}C NMR spectrum was recorded using the CPMAS mode with enhanced resolution. The spectral width was 20000Hz with 4969 transients and a pulse delay of 10s with a pulse width of 6.0 μs.

Solid State ^{13}C NMR of Bilirubin bound to Albumin

The sample was prepared by adding bilirubin (1.35×10^{-5}mol) to an isotonic buffer solution pH 7.2 (10ml) which contained bovine serum albumin (3.62×10^{-5}mol). The resulting solution was incubated for one hour at 37°C while stirring and protected from light. The sample was then freeze dried to remove the solvent. The solid state ^{13}C NMR spectrum of the resulting sample (0.38 mole ratio of bilirubin to albumin) was recorded using the CPMAS mode with enhanced resolution after 668 transients, with a pulse delay of 10s with a pulse width of 6.0 μs.

6.2.3 SOLUTION STATE ^{13}C NMR SPECTROSCOPY

The ^{13}C solution NMR spectrum of 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane in DMSO-d_6 was recorded at 25°C on a Varian CFT-20 spectrometer. The carbon resonance was at 20MHz with a spectral width of 6024Hz, with 82900 transients a pulse width of 7 μs and a pulse delay of 0s. All chemical shifts are quoted relative to TMS.

6.3 RESULTS AND DISCUSSION

6.3.1 STUDY OF THE INTERACTION OF BILIRUBIN AND BOVINE SERUM ALBUMIN BY FLUORESCENCE SPECTROSCOPY

Calculating the Quenching Rate Constant

In the high affinity binding site of bilirubin in bovine serum albumin there are two tryptophan residues. These residues are chromophores, when they absorb light of wavelength 290nm an excited state is formed and the main pathway for dissipation of the absorbed energy is fluorescence. As a result the protein exhibits an intrinsic fluorescence maximum at 350nm. The fluorescence spectrum (Figure 3)

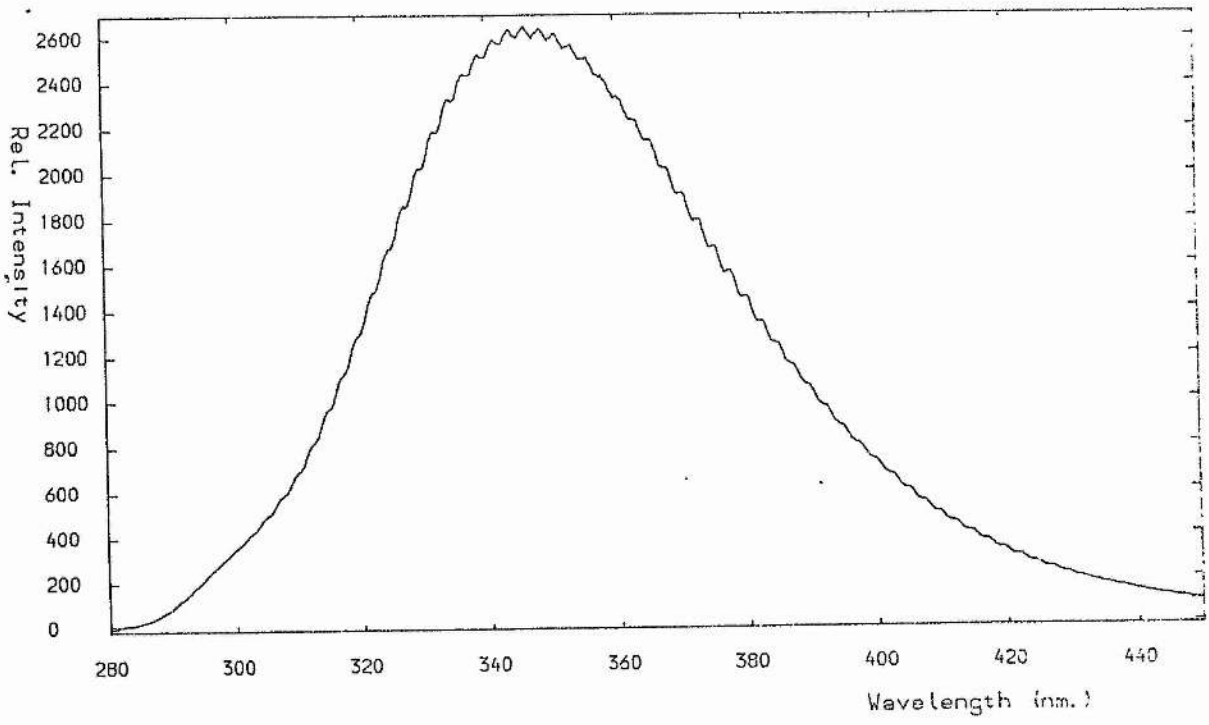


Figure 3 The fluorescence emission spectrum of bovine serum albumin activated at 290nm, in isotonic buffer pH 7.2.

is the sum of the two emitting tryptophyl residues and any energy transfer between them.

The two tryptophan residues are individually characterised by a particular set of physio-chemical conditions that influence the chromophore fluorescence. When a molecule of bilirubin binds to the high affinity site, the microenvironment of the tryptophyl residues is perturbed, which leads to a decrease in fluorescence; i.e. fluorescence quenching. The bilirubin is acting as a quencher molecule, which is capable of absorbing energy, causing a decrease in the number of excited states of the tryptophan, hence reducing the intensity of the fluorescence. There is a physical relationship between the intensity of emission and the concentration of the quencher in solution known as the Stern-Volmer equation :

$$I_e / I_e^\circ = 1 + K_q \tau [Q]$$

where I_e° = emission intensity without quencher

I_e = emission intensity with a concentration [Q] of quencher

K_q = quenching rate constant

τ = lifetime of the excited state

A plot of I_e / I_e against quencher concentration will have a slope of $K_q \tau$, and since τ is constant, the slope is proportional to the quenching rate constant. This procedure was carried out and gave a quenching rate constant of 0.758 for the quenching effect of bilirubin being added to solutions of bovine albumin.

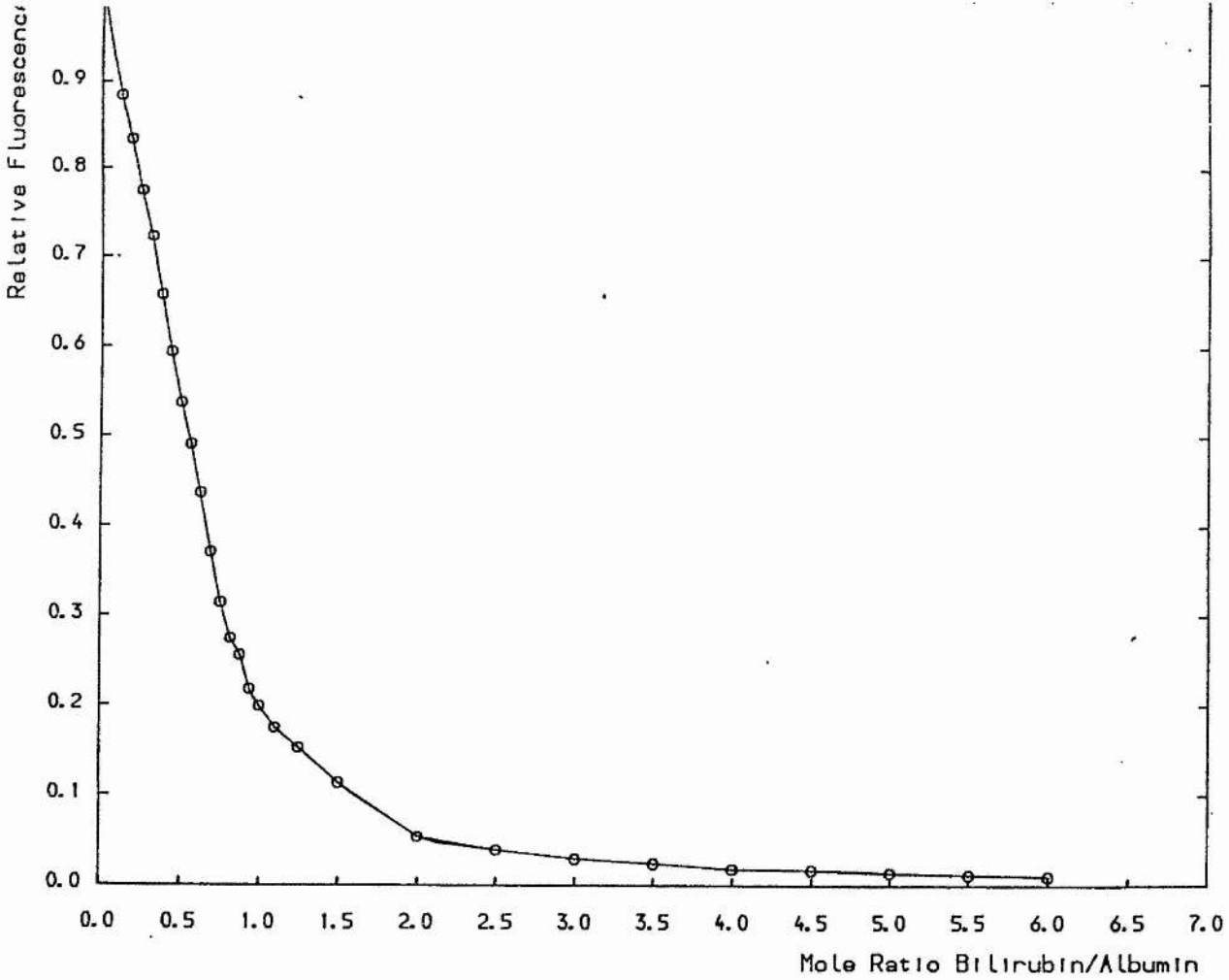


Figure 4 A quench curve titration of bovine albumin emission fluorescence (λ_{max} 350nm) against the mole ratio of bilirubin to albumin.

Determination of Binding Affinity and Capacity

Although the Stern-Volmer equation can be helpful in calculating the quenching rate constant and a linear relationship between quencher concentration and relative fluorescence, the results from these quenching titrations can be better manipulated to determine binding affinity and capacity by employing a Scatchard plot.[25] The full procedure for this is as detailed by Levine.[26] A quench curve approaches a limiting line[27] at lower concentrations of substrate. Therefore, a single titration can be used to determine the binding affinity and capacity. When bilirubin is added to a solution of albumin, and it is completely bound, there is a linear decrease in fluorescence. The quenching plot will be linear at low ratios of bilirubin to albumin. When higher concentrations of bilirubin are present the proportion of unbound bilirubin will increase depending on the affinity constant. Therefore, a quenching curve will be linear at low ratios of bilirubin to albumin, but will deviate upwards from the straight line of the initial portion of the plot (Figure 4). From this plot the slope of the regression line, m_1, was calculated. This was then used to calculate F_1, the fluorescence observed when one molecule of bilirubin is bound to each molecule of albumin, by using the equation;

$$F_1 = F_0 + m_1$$

where F_0 = initial fluorescence of albumin.

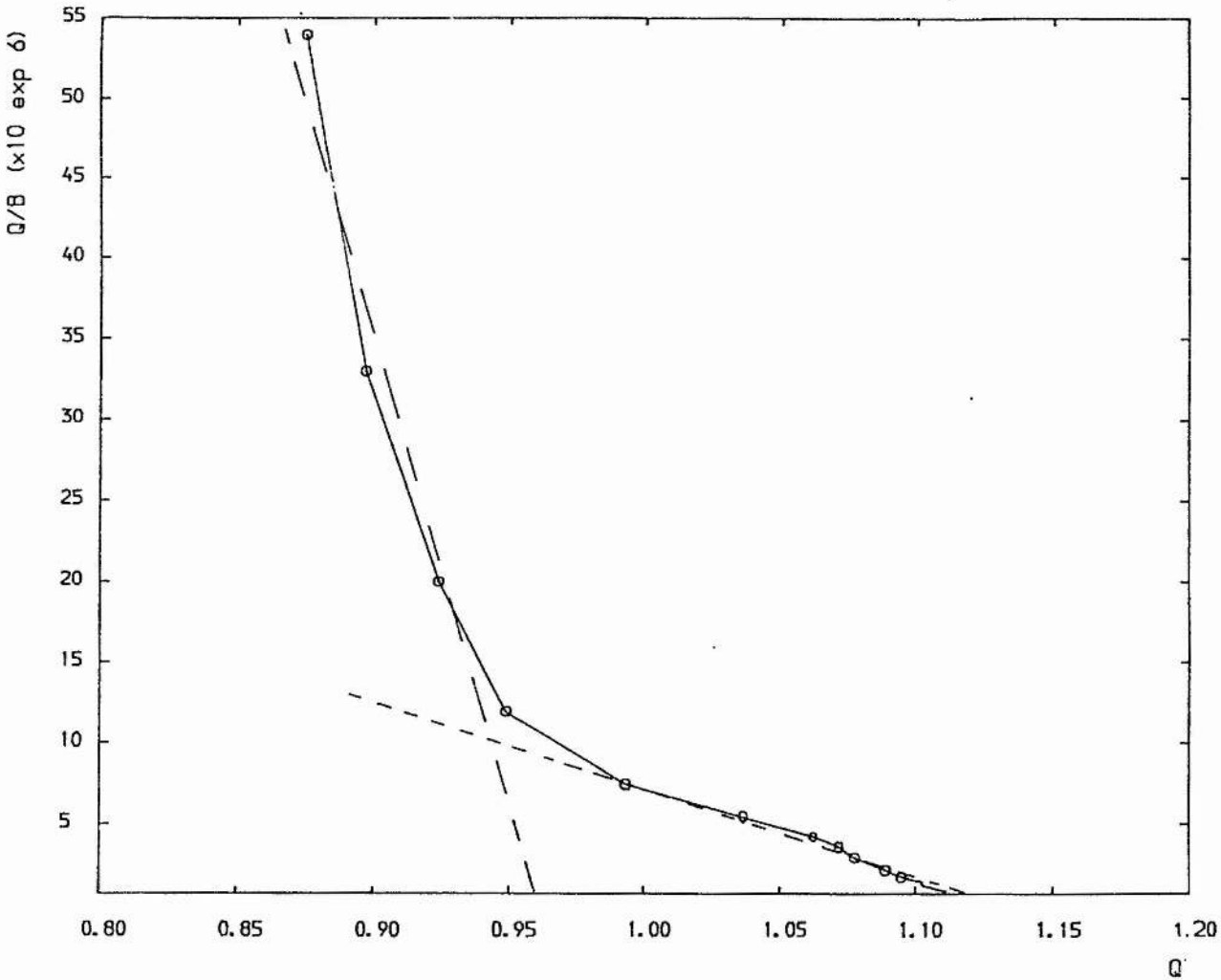


Figure 5 A Scratchard plot for the titration of albumin with bilirubin.

To construct a Scatchard plot, Q, the fractional quench was calculated for every point that deviated upwards from the best fit straight line (Figure 4) using the equation;

$$Q = (F_0 - F)/m_1$$

where F = fluorescence of that point for which Q is calculated.

Then by using the equation;

$$nK_a - K_aQ = Q/B$$

$$= Q/((R-Q).[albumin]_T)$$

where K_a = binding affinity

n = binding capacity

R = mole ratio bilirubin to albumin

B = number of moles of unbound bilirubin

A Scatchard plot of Q against $Q/((R-Q).[albumin]_T)$ (Figure 5) gave a straight line with a slope of $-5.5 \times 10^8 dm^3\ mol^{-1}$, i.e. $-(K_a)$ the affinity constant, and an x intercept of 0.96, i.e. the binding capacity (moles of bilirubin bound per mole of BSA) for the primary binding site of bilirubin. It must be noted that with all empirical methods, when the bilirubin capacity is calculated by the addition of bilirubin there is no well defined end point since the secondary binding site will start being occupied before the first site is saturated. For the secondary binding site the Scatchard plot gave a binding affinity of $5.7 \times 10^7 dm^3\ mol^{-1}$ and a binding capacity of 1.12. These results of binding affinities are slightly higher than most values obtained from various techniques.[28] Chen[27] using fluorescence quenching, calculated the primary binding affinity to be $2.2 \times 10^7 dm^3\ mol^{-1}$ for bilirubin binding to a $10^{-7} mol\ dm^{-3}$ solution of

BSA at an ionic strength of 0.18 mol dm^{-3}, pH 7.4, and at 25°C. The present work was carried out with BSA concentrations of 10^{-5} mol dm^{-3} at pH 7.2 in isotonic buffer and at ambient room temperature (i.e. 20-25°C). Therefore, the conditions of the present experiment were somewhat different from those of Chen.[27] Lee and Gillispie[29] noted dramatic effects on bilirubin bound to BSA in comparison to bilirubin bound to albumin when the pH was changed. The bilirubin bound to BSA is more susceptible to salt changes too, since the bilirubin is thought to bind nearer the surface of the bovine serum albumin. It seems reasonable to assume that the affinity constant (K) will therefore vary with pH etc. This difference in affinity (K) may also be due to different batches varying in their measured affinity for bilirubin or the procedure used for analysis. This is apparent when considering the range of affinities ($K = 10^7$-10^9) obtained for the primary binding site of bilirubin bound to HSA in the literature.[30,31] Levine[26] obtained, by exactly the same procedure, but using different batches of HSA, two binding affinities for bilirubin of 1-2 x 10^8 and 6-7 x $10^7 dm^3$ mol^{-1}.

Therefore, in the present studies the values of the binding affinity (K) obtained using BSA fluorescence quenching to measure both the primary and secondary binding sites seem reasonable to use for further comparisons.

An alternative method of studying the binding of bilirubin to albumin is to measure the enhanced extrinsic fluorescence of bilirubin bound to albumin.[27] When bilirubin in solution is activated at a wavelength of 438nm it shows negligible fluorescence between 460 and

640nm. Upon addition of BSA there is a dramatic increase in the bilirubin fluorescence with a maximum at 533nm (Figure 6).

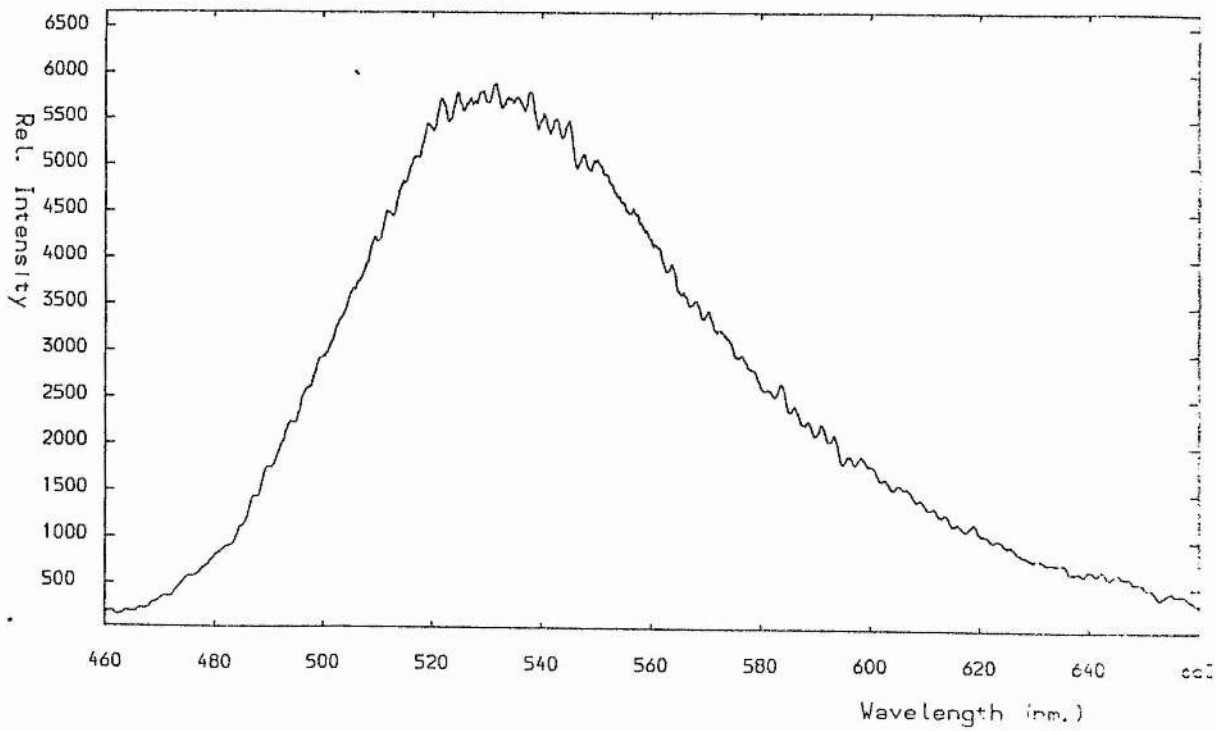


Figure 6 The emission fluorescence spectrum of bilirubin activation at 438nm in isotonic buffer.

When bilirubin is free in solution at pH 7.2 it exhibits extensive intramolecular hydrogen bonding in the molecule (see p. 133). This results in little or no bilirubin fluorescence. In general disruption of the intramolecular hydrogen bonding leads to an increase in the fluorescence of the molecule i.e. it becomes "more open".[29] When bilirubin is bound to albumin the bilirubin intramolecular hydrogen bonds have been destroyed leading to a vast increase in bilirubin fluorescence due to a "more open" species.[29] This enhanced

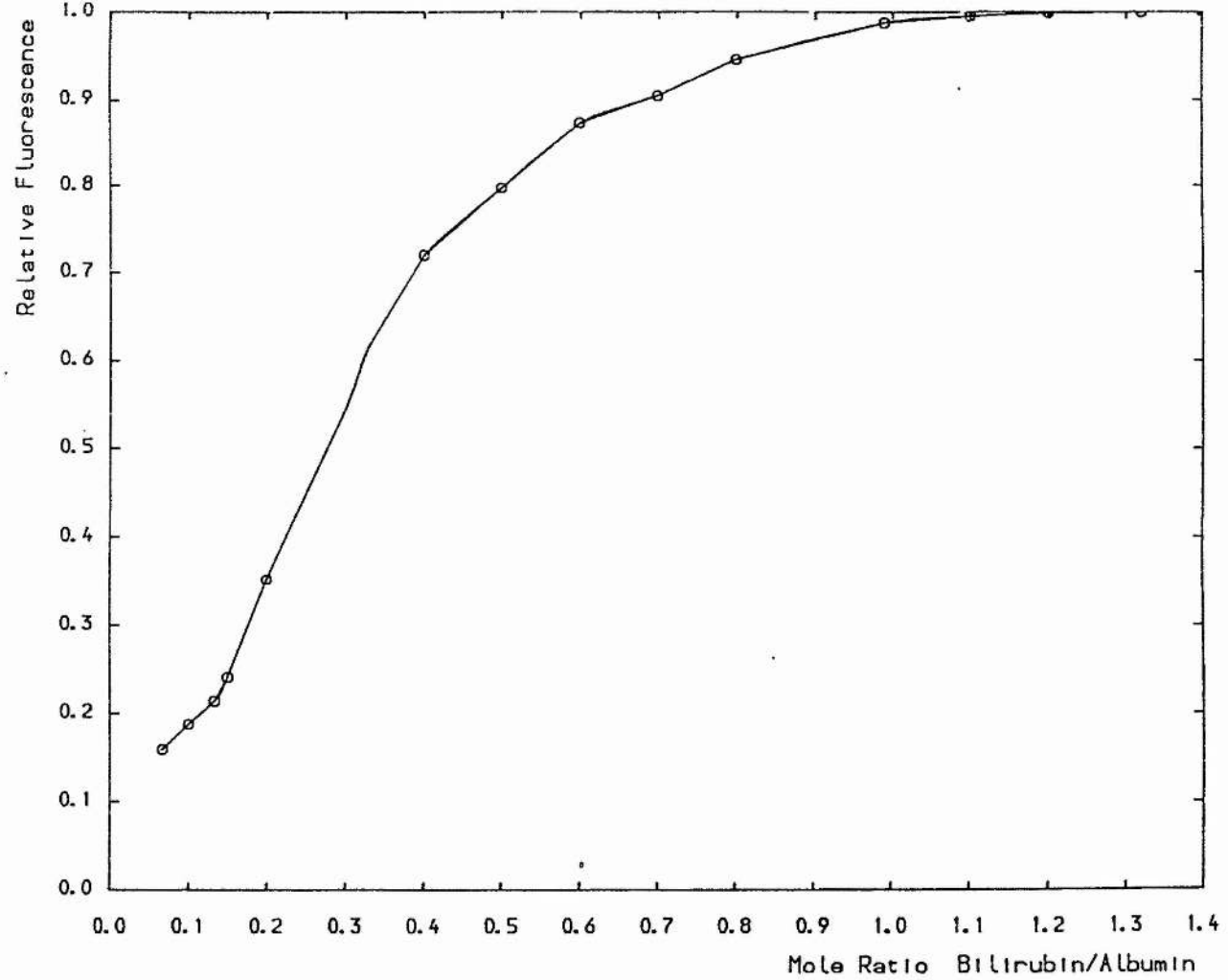
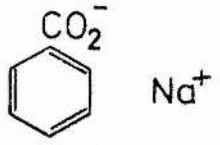
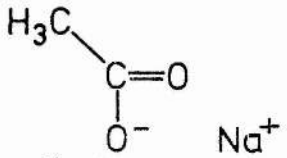


Figure 7 Plot of bilirubin emission fluorescence (λ_{max} 533nm) against the mole ratio of bilirubin to albumin.

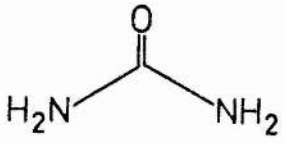
Caffeine



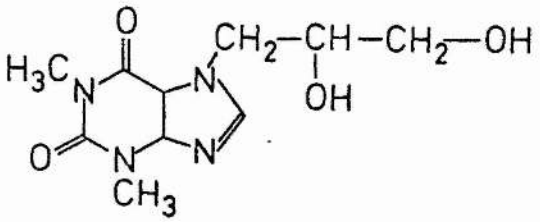
Sodium Benzoate



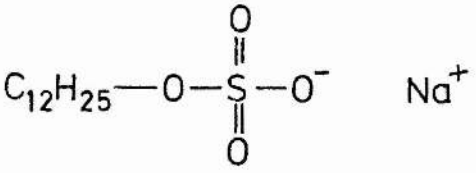
Sodium Acetate



Urea



Dyphylline



Sodium Dodecyl Sulphate
(SDS)

Figure 8

fluorescence can be employed to directly monitor the bilirubin to albumin binding. This is demonstrated in Figure 7 where the bilirubin emission fluorescence is plotted against the mole ratio of bilirubin: albumin. The enhanced emission fluorescence titration is comparable with Figure 4; the quenching fluorescence titration of bilirubin to albumin.

Using the bilirubin enhanced fluorescence titration curve (see Figure 7) the primary binding site was calculated by a Scatchard plot to give a primary binding affinity of $1.6 \times 10^{8} dm^{3} mol^{-1}$. This value is comparable with that of $5.5 \times 10^{8} dm^{3} mol^{-1}$ for the primary binding affinity calculated by measuring tryptophan quenching.

6.3.2 STUDYING THE EFFECT OF "ACCELERATORS" ON THE BINDING OF BILIRUBIN TO ALBUMIN

Jendrassik and Grof[21] first reported that using a mixture of caffeine and sodium benzoate could "accelerate" the formation of azobilirubin in the "indirect" reaction van den Bergh test (see Chapter 5 p. 138). This method of total bilirubin measurement has been recommended by the National Committee for Clinical Laboratory Standards (NCCLS, USA), and is routinely employed today. There have been many modifications of the original Jendrassik-Grof method and a large variety of different "accelerators" recommended[33] (Figure 8). Michaellson[34] proposed the use of dyphylline instead of caffeine to avoid turbidity, Watanabe *et al*[35] recommend sodium dodecyl sulphate

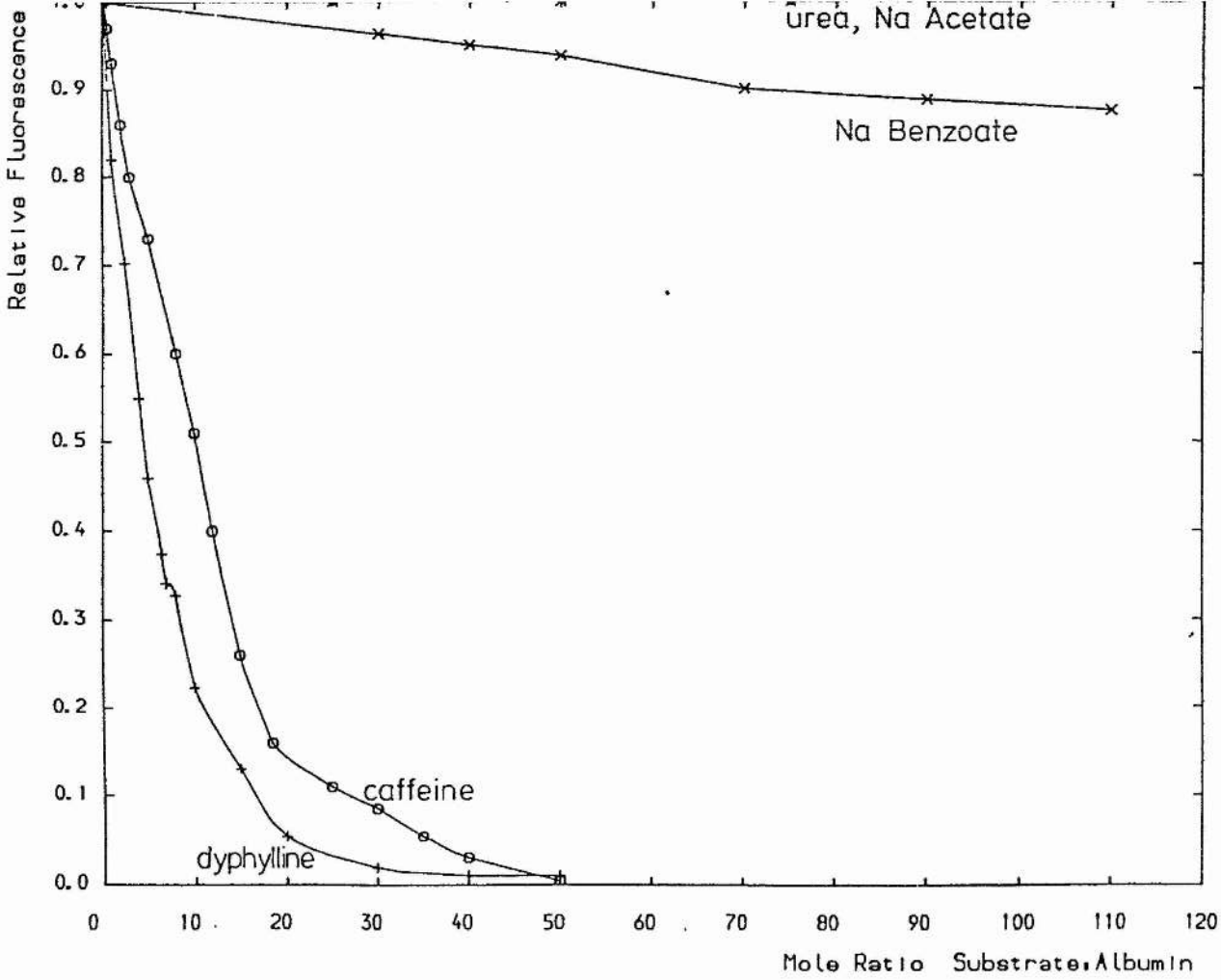


Figure 9 Plot of albumin emission fluorescence (λ_{max} 350nm) against the mole ratio of "accelerator" to bilirubin.

(SDS) as an alternative accelerator. Landis and Purdue[36,37] have studied the kinetics of the "indirect" reaction with diazotised sulphanilic acid using caffeine and sodium benzoate as accelerators. Nevertheless, the question why these different accelerators work and their mode of action has never been adequately answered. It is thought that accelerators might alter the bilirubin-albumin binding in some manner, rendering the bilirubin more accessible to electrophilic attack by the diazotised sulphanilic acid. These accelerators may exhibit competitive binding to albumin, where in one albumin molecule bilirubin and accelerator cannot both be bound simultaneously. Alternatively, they may bind non-competitively, where both substrates can be bound to the same molecule of albumin, but the binding affinity of both the accelerator and the bilirubin is decreased due to conformational changes of the albumin.

Bilirubin emission coupled with tryptophan emission fluorescence was used to study the effect of various accelerators and mixtures of accelerators on the binding of bilirubin to albumin.

The Effect of "Accelerators" on the Albumin Tryptophan Emission Fluorescence

Several accelerators were titrated with BSA and their quenching effect on the tryptophan emission fluorescence monitored (Figure 9). Sodium acetate and urea both had no effect on the tryptophan emission. The fluorescence was not quenched at all, nor was the emission maximum shifted from 355nm by even mole ratios of urea or sodium acetate to albumin of $\geq 25 \times 10^3$ (not shown in Figure 9). Sodium

dodecyl sulphate (SDS) is an anionic surfactant which can bind to proteins. This results in the protein having an overall negative charge and can cause complete denaturation of the protein. When SDS was added to the albumin solution the emission fluorescence maximum shifted from 355nm to 315nm. This indicates that the tryptophan microenvironment has been dramatically perturbed. Since the protein secondary structure has been destroyed the quenching effect of SDS on the actual binding site of bilirubin cannot be measured. A mole ratio of 13.6×10^2 SDS-albumin decreased the emission fluorescence at 315nm by $\sim$50% relative to the initial fluorescence of albumin. When SDS addition to the system was further continued the tryptophan fluorescence showed no further quenching.

When aliquots of sodium benzoate were added to albumin solutions, at low mole ratios of benzoate to albumin there was no significant quenching of the tryptophan emission. Only at mole ratios of $\geq 100:1$ did the benzoate exhibit an appreciable quenching effect. The Scatchard plot method under these experimental conditions gave a binding affinity of 7.47×10^4 dm^3 mol^{-1}. In comparison with the binding affinity of bilirubin ($5.5 \times 10^8 dm^3$ mol^{-1}), the binding affinity of benzoate is almost 10^4-fold lower.

Caffeine and dyphylline both showed a reasonable quenching effect on the tryptophan emission at relatively low mole ratios of substrate to albumin (Figure 9). Caffeine was calculated by the Scatchard plot method to give a binding affinity of $2.4 \times 10^6 dm^3$ mol^{-1}, the corresponding value for dyphylline being $2.9 \times 10^6 dm^3$ mol^{-1} under these experimental conditions. The difference in binding affinity (K) may

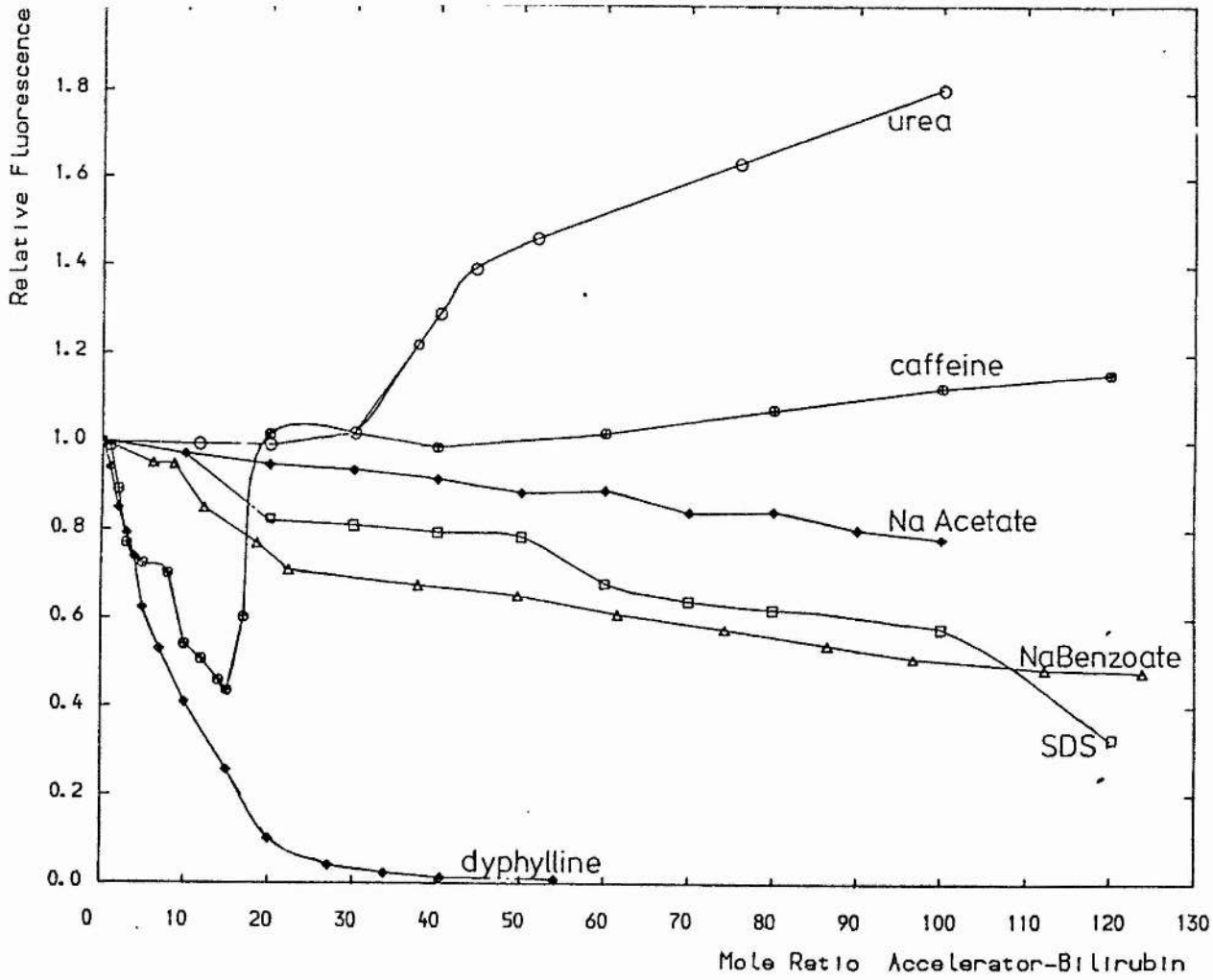


Figure 10 Plot of bilirubin emission fluorescence (λ_{max} 533nm) against the mole ratio of "accelerator" to bilirubin.

be due to solubility differences between the two molecules. In the Jendrassik-Grof method sodium benzoate is apparently used to increase the solubility of caffeine by the formation of an association complex.[38]

Studying the Effect of "Accelerators" on the Binding of Bilirubin to Albumin using Bilirubin Emission Fluorescence Quenching

To determine whether any of the accelerators have an effect on the actual binding of bilirubin to BSA the change in the bilirubin emission was monitored as varying amounts of accelerators and mixtures of accelerators were added to the bilirubin-BSA system. Initially the bilirubin emission fluorescence was recorded of a solution containing a 0.73 mole ratio of bilirubin to albumin, this gave a maximum relative fluorescence (1.0) where the bilirubin is tightly bound to the primary binding site. Then aliquots of accelerator were added and after each addition the bilirubin fluorescence recorded. Any change in fluorescence can be directly attributed to changes in the binding of the bilirubin (see p. 149) bound to albumin. Therefore, a decrease in binding affinity would lead to directly to a decrease in bilirubin fluorescence.

The relative fluorescence was plotted against the mole ratio of substrate to bilirubin (see Figure 10) and the mole ratio of substrate to bilirubin required to decrease the bilirubin fluorescence by 50%, i.e. $F_{1/2}$, was noted (see Table 1).

Table 1

Accelerator	$F_{1/2}$
Sodium Benzoate	100
Caffeine	12
Dyphylline	7
SDS	100
Sodium Acetate	—
Urea	—

$F_{1/2}$ values quoted as mole ratio of substrate to bilirubin.

When sodium benzoate was added in small aliquots to bilirubin tightly bound to BSA, there was a very gradual decrease in the emission fluorescence with $F_{1/2}$ reached only when a 100-fold excess of benzoate was added. Therefore, sodium benzoate alone can act as an accelerator, but rather large mole ratios of benzoate to bilirubin are required. Benzoate does not compete with bilirubin at the same binding site, but gradually decreases the capacity of albumin for bilirubin by conformationally changing the protein. Similarly, the anionic surfactant SDS caused a 50% decrease in bilirubin fluorescence at approximately a 100-fold excess of SDS to bilirubin. This result can be explained by the mode of binding action of SDS to the BSA surface. SDS indiscriminantly binds to the protein surface and this gradually results in conformational changes to the protein which may result in denaturation. The action of SDS destroys the high affinity binding site of bilirubin, therefore SDS can be classed as a non-competitive bilirubin binding inhibitor. Sodium acetate addition

resulted in no significant decrease in bilirubin emission fluorescence up to a mole ratio of 100:1 acetate to bilirubin. When this ratio was increased to 1000:1 there was only a 25% decrease in the total relative fluorescence. Hence, sodium acetate can not only be considered to be non-competitive, but ineffective in removing bilirubin from its high affinity binding site in BSA at mole ratios of $\geq$ 1000:1 acetate to bilirubin.

In contrast both dyphylline and caffeine exhibited marked changes in bilirubin binding at low substrate to bilirubin mole ratios (see Figure 10). In the case of dyphylline $F_{1/2}$ was observed at an 8-fold excess of dyphylline. When aliquots of caffeine were added to the bilirubin-albumin system $F_{1/2}$ was reached at a caffeine to bilirubin mole ratio of 12-13:1. As addition of caffeine continued this lead to an apparent increase in bilirubin fluorescence, whereas with dyphylline the fluorescence decreased to a minimum. This apparent increase is actually light scattering, which is caused by unbound bilirubin under these conditions exceeding its solubility leading to colloid formation and hence light scattering. This is quite commonly observed when studying the binding affinity and capacity of bilirubin to small concentrations (0.4μmol dm^{-3}) of albumin and when monitoring the effects of drugs on the binding of bilirubin to albumin by the technique of fluorescence spectroscopy.[26]

The results obtained from the addition of the accelerator urea (see Figure 10) cannot be easily explained, there tended to be no significant decrease in fluorescence due to the addition of urea, then at a urea to bilirubin mole ratio of 30-40:1 an increased fluorescence

was observed. This again may be light scattering due to bilirubin colloid formation, but there seemed to be no initial decrease in fluorescence before light scattering is observed. Therefore, no conclusions can be made about the ability of urea to decrease the binding of bilirubin to BSA.

Several different accelerators have been investigated by Lolekha and Limpavithayakul[39] to measure the ability of these accelerators to promote the "indirect" diazo reaction of bilirubin. Using visible spectroscopy they measured the increased absorption at 600nm due to the formation of azobilirubin. The accelerators investigated were sodium acetate, HCl, dyphylline, sodium benzoate, caffeine, and mixtures of these.

As an accelerator they found caffeine alone could promote increased formation of azobilirubin when bilirubin was bound to BSA. This is in keeping with the ability of caffeine to decrease effectively the capacity of albumin for bilirubin, as found by this present work. Using various amounts of bilirubin (20-230mg dm^{-3}) bound to BSA and HSA these workers recommended 9g dm^{-3} of caffeine, or this amount in a combined mixture with other accelerators, to promote the reaction. When this recommended 267-fold excess of caffeine to bilirubin was added to the same bilirubin-albumin system as before the bilirubin emission fluorescence gave a value of 1.163 (see Table 2) hence bilirubin had effectively been removed from the binding site and formed an insoluble colloid. However, from the previous fluorescence studies bilirubin could have been effectively removed by mole ratios of only 20-50:1 and hence allow the reaction of unbound bilirubin and

sulphanilic acid to take place. It is astonishing to find that in many methods caffeine concentrations of 15-50g dm^{-3} are used.[40] Similarly, Lolekha and Limpavithayakul found dyphylline to be a good accelerator and suggested it be used up to a maximum concentration of 10g dm^{-3} itself, or more effectively with a combination of accelerators. This recommended amount is a 226-fold excess of dyphylline to bilirubin. In contrast the present work indicates that $\leqslant$ 50-fold excess of dyphylline is more than adequate to remove bilirubin from its high affinity binding site (see Figure 10).

From this present study sodium benzoate was found to decrease gradually the capacity of BSA for bilirubin, but a $\geqslant$ 140-fold excess of benzoate to bilirubin would be required to remove successfully the majority of the bilirubin from the protein. Lolekha and Limpavithayakul suggest up to a 2170-fold excess as being effective. This again seems far too great an excess to adequately remove bilirubin from BSA. Sodium acetate was found by these workers not to accelerate the "indirect" reaction of bilirubin bound to BSA with diazotised sulphanilic acid. They recommend sodium acetate not to be used alone as an accelerator, but only in combination with other agents at concentrations of 90g dm^{-3}. This is indeed similar to the effect of sodium acetate on the binding of bilirubin to BSA. When sodium acetate was added up to 1000-fold excess (not shown in Figure 10) the relative fluorescence had only dropped to 0.75. Alternatively, when sodium acetate was added in 7350-fold excess (as recommended by Lolekha and Limpavithayakul) there was an increase in the relative fluorescence to 1.128 (see Table 2) indicating bilirubin colloid formation. At this excessive acetate to bilirubin mole ratio sodium

Table 2

	Accelerator	Mole Ratio Accelerator/Bilirubin	Relative Fluorescence
(i)	caffeine	267	1.163
(ii)	sodium acetate	7350	1.128
(iii)	urea	1000	1.720
(iv)	caffeine + urea	267 + 1000	1.070
(v)	caffeine + acetate	267 + 7350	0.879
(vi)	acetate + urea	7350 + 1000	0.825
(vii)	caffeine + urea + acetate	267 + 1000 + 7350	0.804

acetate must be denaturing the albumin and hence releasing the bilirubin from the binding site.

The effect of urea as an accelerator is difficult to interpret. The urea does not appear to have any significant effect except addition causes extensive light scattering. When a 1000-fold excess of urea to bilirubin, as recommended by Lolekha and Limpavithayakul, was added to the bilirubin-albumin system the resulting relative fluorescence indicated extensive light scattering (see Table 2).

As a result of their studies Lolekha and Limpavithayakul proposed a combination of accelerators as an effective accelerator mixture (i.e. 9g caffeine, 10g urea, and 90g sodium acetate in 1l) to promote the reaction of "indirect" bilirubin with diazotised sulphanilic

acid. As discussed previously these amounts of accelerator were investigated by fluorescence spectroscopy to determine their ability to remove bilirubin from the high affinity binding site of BSA. Then different combinations of these relative amounts were added to the bilirubin-albumin system and finally the mixture itself was assessed as a bilirubin binding inhibitor. The resulting relative fluorescence values are shown in Table 2. The actual mixture was shown to be slightly more effective than the different combinations of two accelerators, but the mixtures were not as effective as the accelerators by themselves in removing the bilirubin from the BSA to the extent that a bilirubin colloid is formed. Possibly this proposed mixture does not accelerate the release of bilirubin from albumin to any greater degree but instead increases the rate of the reaction of free bilirubin with diazotised sulphanilic acid.

In conclusion, this present study has successfully evaluated the effect of various so called "accelerators" on the "indirect" bilirubin reaction to remove bilirubin from its high affinity binding site on BSA. None of the accelerators studied exhibited competitive binding at the bilirubin high affinity binding site. Some accelerators more than others were successful in decreasing the capacity of the BSA for bilirubin, and hence were non-competitive inhibitors of bilirubin binding. From these present results it is apparent that both caffeine and dyphylline can be independently used as successful non-competitive inhibitors but at much lower concentrations, e.g. 1.81g dm^{-3} (a 50-fold excess of accelerator to bilirubin), rather than the 9-10g dm^{-3} (267-fold excess) recommended by previous workers. This work did not find caffeine any more effective in a combination mixture with

sodium acetate and urea. Sodium benzoate independently is not a very effective inhibitor at low ratios of benzoate to bilirubin, therefore, in the Jendrassik-Grof method benzoate is clearly used to increase the solubility of the caffeine, hence increasing its accelerating ability. SDS must be added in large quantities ($\geq$ 100-fold excess) to denature effectively the BSA thus releasing bilirubin and hence it is not as effective as caffeine or dyphylline. Sodium acetate cannot act as an accelerator alone, only at extreme mole ratios of acetate to bilirubin (e.g.$\gtrsim$7350) does this inhibit bilirubin binding fully. Acetate must be used in combination with other accelerators, if at all. The action of urea is not fully understood, but it seems to effectively remove bilirubin at much lower concentrations than previously recommended (i.e. $\sim$1000-fold excess). In the "indirect" reaction perhaps the role of urea and sodium acetate in such vast excess is to decrease the binding of bilirubin by changing the pH of the system so drastically so that the protein is denatured and thus bilirubin is released. Bilirubin-BSA binding is much more susceptible to pH changes than bilirubin-HSA binding. In addition albumin at physiological pH is in the N-form conformation which readily binds bilirubin, but at pH 12 the C-form conformation exists which does not bind bilirubin. Likewise, at extremely low pH (pH $<$ 4) albumin exists in a third conformation, the F-form. Very little research has been carried out on the F-form of albumin, but it seems that bilirubin does not bind to this form either.

6.3.3 THE EFFECTS OF POLYAMINO ACID CHAINS-ON BILIRUBIN EMISSION FLUORESCENCE

Trull et al[41] first reported bilirubin binding by covalent linkage to a polystyrene matrix polymer. This work indicated that simple polymer matices could be good models for bilirubin binding proteins. Since the bilirubin binds covalently to the polymer, this binding action is appropriate to study, thus revealing information on the interaction of bilirubin with more complex systems, i.e. bovine serum albumin. More recently Marr-Leisy et al[22], using induced circular dichroism, reported bilirubin binding to a polyamino acid; poly-L-lysine, at pH 11.4. Using the technique of bilirubin emission fluorescence the binding of bilirubin to poly-L-lysine was initially studied.

At pH 11.4 bilirubin is much more soluble than at pH 7.2. For the bilirubin molecule, the two carboxyl and two lactam moieties have pK values in water of 4.4, 4.4, 13.0, and 13.0 respectively.[42] Therefore, at pH 11.4 both the carboxylic acids will be fully deprotonated and a higher percentage of lactam moieties are dissociated in comparison to pH 7.2. This degree of deprotonation leads to destruction of the intramolecular hydrogen bonding of the bilirubin leading to increased solubility. The bilirubin emission fluorescence was recorded for a solution of bilirubin (1.04×10^{-5} mol dm^{-3}) at pH 11.4, then aliquots of poly-L-lysine were added and the resulting fluorescence recorded. Relative fluorescence was plotted against the mole ratio of bilirubin to poly-L-lysine. This gave a fluorescence enhancement

titration which was subjected to a Scatchard plot. The affinity constant, K, for bilirubin binding to poly-L-lysine (α helix) was calculated to be 2.2 x $10^6 dm^3\ mol^{-1}$ with a binding capacity of 0.96 for one mole of poly-L-lysine. Therefore, bilirubin has a 100-fold lower binding affinity for poly-L-lysine at pH 11.4, than for the primary binding site of BSA at pH 7.2. Nevertheless, this degree of affinity for poly-L-lysine indicates the significance of lysine residue 240 in the primary binding site of HSA. The amino acid lysine has a flexible side chain with a potentially reactive amino acid group at the end. The high pK_a of $\sim$10.5 means that the side chains are protonated at physiological pH. Jacobsen[12] found carbodi-imide coupling experiments that bilirubin is bound to lysine 240. Therefore, it is likely that one of the carboxylate groups of bilirubin binds to the lysine by an electrostatic interaction, as well as by hydrophobic interactions with other amino acids of the primary site.

At pH 11.4 the α helix poly-L-lysine unit will have deprotonated amino acid chains and hence be over all basic in nature. In the same instance some bilirubin, as discussed previously, although soluble at pH 11.4 it exhibits negligible fluorescence. On addition of the basic poly-L-lysine the bilirubin emission fluorescence was enhanced since the intramolecular hydrogen bonds between the lactams and the propanoic acid groups are disrupted leading to an electrostatic interaction with the propanoic acid groups and the basic side chains of the poly-L-lysine. Bilirubin is now in a more open form which is an active chromophore.

Marr-Leisy *et al*[22] reported that when a random coil of poly-lysine was used instead of poly-L-lysine, even although the side chain amino acid would be basic, there was no significant interaction with bilirubin at pH 11.4. Hence the conformation of the α helix (i.e L form) of poly-lysine is vital. The particular orientation of the side chain amino acids and their hydrophobic effects, in addition to their electrostatic interaction with bilirubin accounts for the a binding affinity of $2.2 \times 10^{6} dm^{3} mol^{-1}$.

The effect of a poly-L-asparagine amino acid chain on the bilirubin emission fluorescence was then investigated at pH 7.2. When poly-L-asparagine ($5.5 \times 10^{-4} mol\ dm^{-3}$) was added to a solution of bilirubin ($1.05 \times 10^{-5} mol\ dm^{-3}$) there was a 1.68-fold increase in the fluorescence. This enhanced bilirubin fluorescence can be explained if the characteristics of the amino acid asparagine are considered. Asparagine has a amide group side chain which is not acidic, but is polar and able to participate in hydrogen bonding. Therefore, poly-L-asparagine is a polar amino acid chain which, when introduced into a system with only negligible fluorescence produces an increase in fluorescence. This is due to a more open bilirubin chromophore that binds to the poly-L-asparagine by hydrogen bonding as well as by hydrophobic interactions. These bilirubin interactions with poly-L-asparagine, although not as dramatic as those with poly-L-lysine at pH 11.4, must be thermodynamically more favourable than bilirubin intramolecular hydrogen bonding at pH 7.2. The amino acid asparagine is not found at the high affinity binding site of

Table 3

Solution State	Solid State	Assignment
162.37	168.295	10, 15
	164.993	
130.60	134.949	9, 1
	132.618	
125.31	129.640	4, 6
	128.539	
117.02	117.143	2, 8
115.24	115.136	3, 7
21.78	20.149	5
10.25	12.119	11, 14
	10.953	
8.58	9.723	12, 13

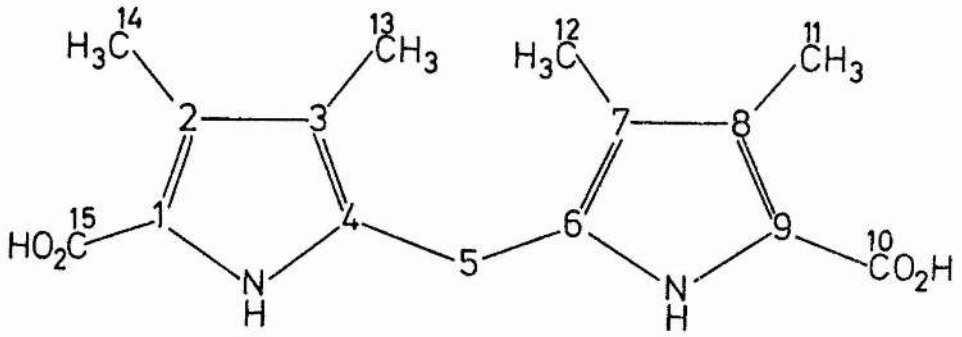


Figure 11 3,3',4,4'-Tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane

bilirubin. However, this study has indicated that at pH 7.2 hydrogen bonding and hydrophobic interactions with the asparagine chain are strong enough to destroy the intramolecular hydrogen bonding of bilirubin.

6.3.4 AN INVESTIGATION OF THE INTERACTION OF BILIRUBIN AND BSA BY THE TECHNIQUE OF ^{13}C SOLID STATE NMR SPECTROSCOPY

The ^{13}C Solid State NMR Spectrum of Dipyrrylmethane

Due to problems of solubiltiy using ^{13}C solution state NMR the interaction of bilirubin and BSA was further investigated by the technique of ^{13}C solid state NMR. Initially, to establish the correct ^{13}C solid state assignments of bilirubin a simple model dipyrrole, 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane (Figure 11) was investigated using CPMAS and TOSS modes of ^{13}C solid state NMR. These assignments were obtained by comparison with the ^{13}C solution state NMR spectrum (see Table 3 and Figures 11 and 12).

It is obvious, by comparison, that the dipyrrylmethane has different orientations in solution and solid states. In the solution state several sets of carbons share identical chemical shifts and hence chemical environments, e.g. C_{10}, C_{15}; C_{11}, C_{14}; C_9, C_1; etc, whereas, in the solid state they have distinctive chemical shifts and hence different microenvironments in the crystalline lattice. There are also specific changes in the chemical shifts of several of the carbon resonances. The two carboxylic acid carbons have moved downfield from their chemical shifts in solution by 2 and 6ppm,

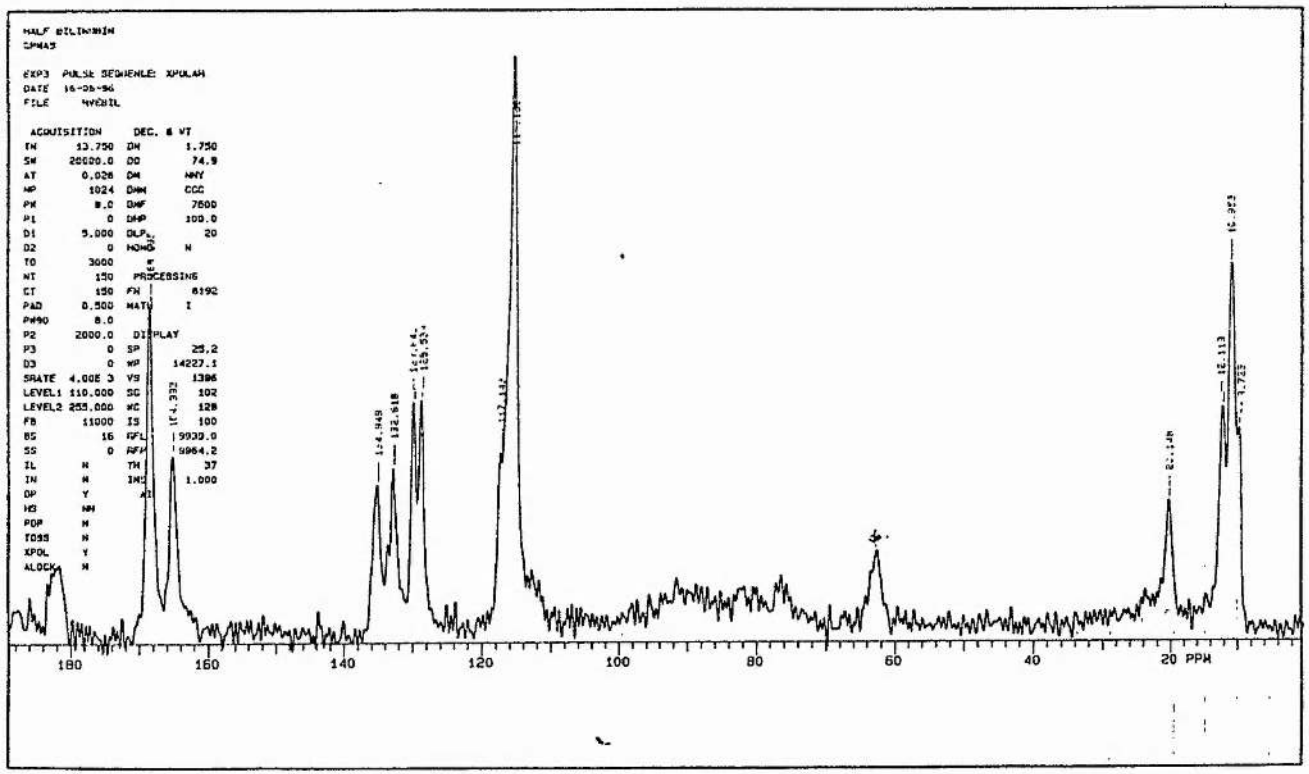


Figure 12 ^{13}C solid state NMR spectrum of 3,3',4,4' tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane using CPMAS and TOSS modes.

implying both have become more electronegative than in solution. In the crystal packing of the dipyrrylmethane the two carboxylic acids must be involved in intermolecular hydrogen bonding and electrostatic interactions with other molecules. In addition C_9, C_1, C_4, C_6, and C_5 within the pyrrole rings exhibit a downfield chemical shift in the solid state due to intermolecular interactions, whereas the other carbons of the pyrrole rings, C_2, C_8, C_3, and C_7, have exactly the same chemical shifts in both solution and solid state. It is apparent that these carbons do not significantly interact with other molecules to any degree when close packed in the crystal lattice since they do not have any hydrogen atoms attached which participate in intermolecular hydrogen bonding. The methyl carbons, C_{12}, C_{13}, C_{14}, and C_{11}, in the solid state show changes in chemical shift again indicating intermolecular interaction, possibly hydrogen bonding and a different orientation in the crystal.

The ^{13}C Solid State NMR Spectrum of Bilirubin IXα-z,z

There have been a large variety of investigations into the ^{13}C NMR assignments of bilirubin IXα-z,z and the behaviour of the molecule in solution.[43,44] Nevertheless, up until 1985 when Muller[45] reported the full assignment of the ^{13}C NMR spectrum using two dimensional heteronuclear ^{1}H-^{13}C chemical shift correlation[46], there had been no complete assignment of the ^{13}C resonances of the ring carbons in the molecule.

In the solid state bilirubin crystals exist in a triclinic form.[47] There are two enantiomeric molecules of bilirubin[48] in the crystal structure, both have z,z configuration at the C_5 and C_{15} bridges. The two enantiomers have very similar ridge tile confor-

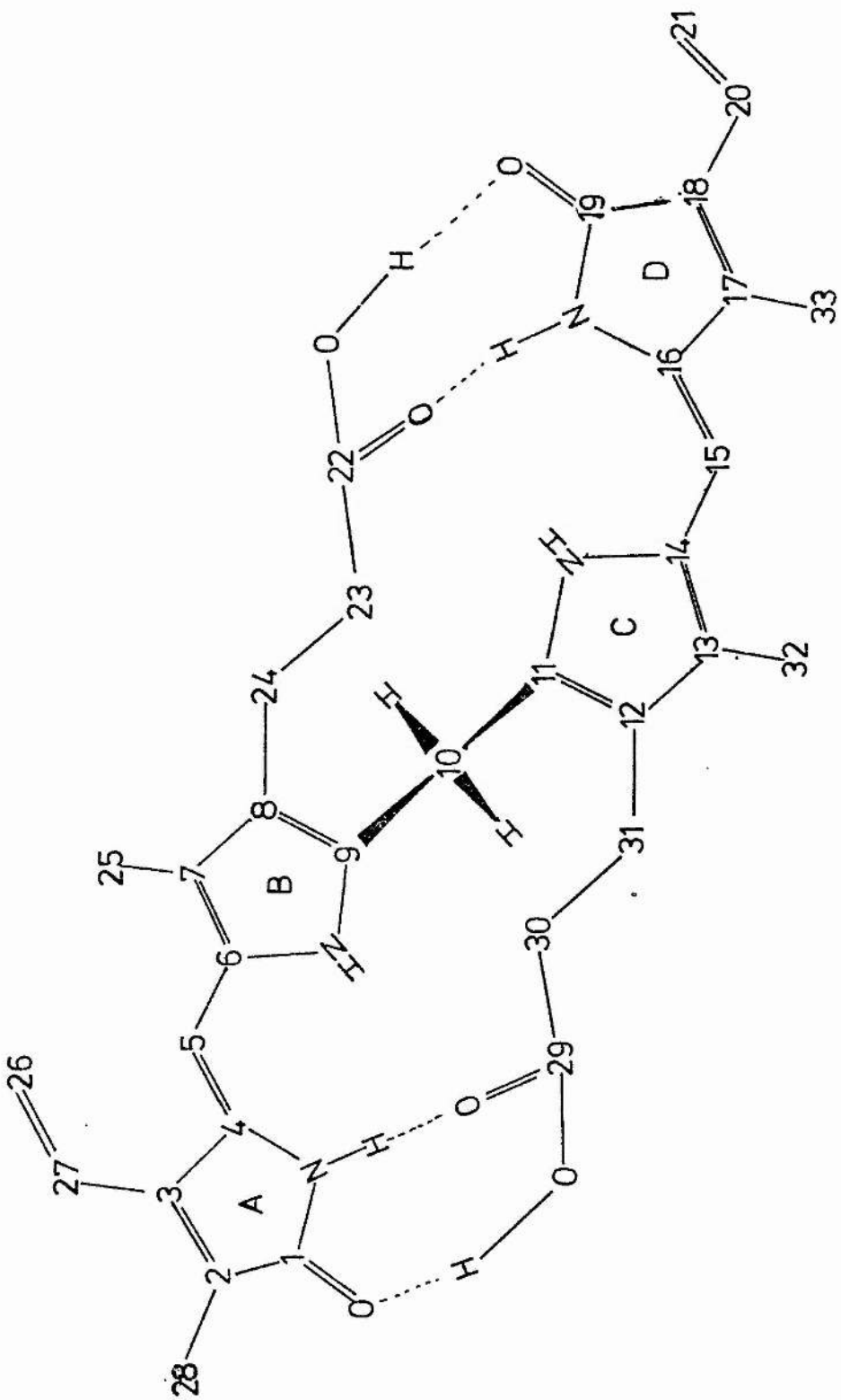


Figure 13 Bilirubin IX α-z,z

mations (see Photographs 21 and 22). In this ridge tile conformation the two planes, with an interplanar angle of approximately 97°, are formed by rings A + B and C + D. Six intramolecular hydrogen bonds stabilise each of the bilirubin molecules (see Figure 13) in the crystal structure, but there is no evidence of intermolecular hydrogen bonding. Bilirubin can be regarded as a 2,2'-dipyrrylmethane which at the α positions has conjugating substituents since the bond lengths suggest that delocalisation over the two individual conjugated pyrrole systems is limited, i.e. C_5-C_6, and C_{14}-C_{15} resemble single bonds, whereas C_{15}-C_{16}, and C_4-C_5 are essentially double bonds. In the crystal packing stacks of these ridge shaped bilirubin molecules run in parallel lines and are interlocked with inverted stacks. In the stacking lines adjacent molecules are enantiomers.

In solution state, e.g. in chloroform, bilirubin has been found by ^{1}H NMR studies[49] to possess these six distinctive intramolecular hydrogen bonds with z,z configuration at C_5 and C_{13}. In solution the bilirubin molecule exists as two enantiomeric conformers, the thermodynamics of the interconversion (i.e. the breaking of the six intramolecular hydrogen bonds and rotation about the central methylene bridge) has been studied by Navon *et al.*[48] Each of the enantiomers, as in the solid state, exhibits no intermolecular bonding.

Due to problems of solubility all the ^{13}C solution NMR had to be run in DMSO-d_6, which destroys any intramolecular hydrogen bonding.

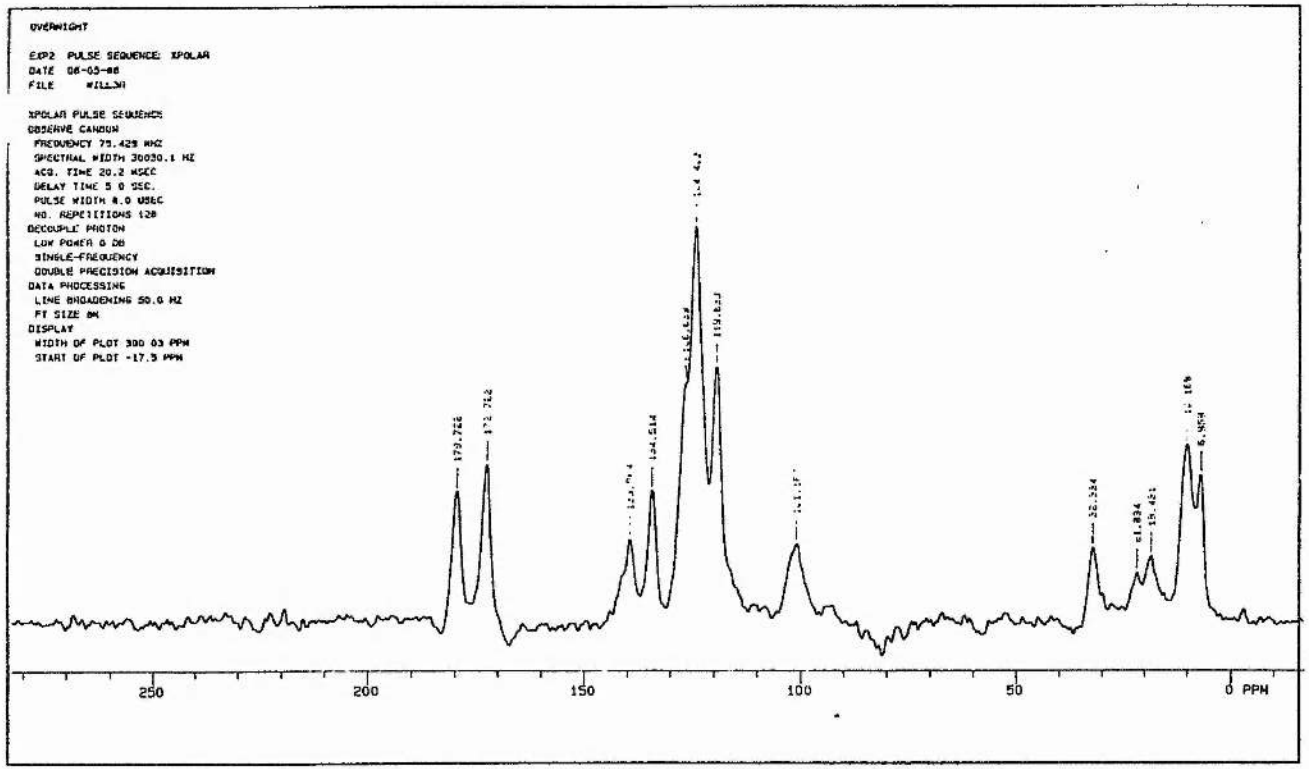


Figure 14 ^{13}C solid state NMR spectrum of bilirubin IXα-z,z using CPMAS mode with enhanced resolution.

In the light of the characteristics of bilirubin in both solution and solid state discussed previously, the ^{13}C assignments in solution state (DMSO-d_6) and solid state were compared (see Table 4 and figures 13 and 14). Although, in solid state the ^{13}C resonances are not as well resolved as by the technique of 2D NMR, nevertheless the effect of intramolecular hydrogen bonding on the ^{13}C resonances can be directly observed. This is very apparent in specific ^{13}C resonances; C_{22}, C_{29}, the carbons of the carboxylic acid groups have shifted from 174.1ppm in solution, where they are hydrogen bonded to the DMSO, to 179.7ppm. This downfield chemical shift of 5ppm can be directly attributed to intramolecular hydrogen bonding with the pyrrole imino hydrogen atoms. C_1 and C_{19} of the terminal lactam systems exhibit a slight downfield shift of 2 and 1ppm respectively due to hydrogen bonding with the OH group of the carboxylic acid groups. These intramolecular hydrogen bonding effects are noted to a lesser extent in other carbon shifts, e.g. the α (C_{23}, C_{30}) and β (C_{24}, C_{31}) carbons of the carboxylic acid group show a slight upfield shift of 2 and 1ppm respectively. Other slight chemical shift changes may be due to differences in orientation and steric effects in solution and solid state, e.g. in the solid state a ridge tile and in DMSO a more open structure due to solvation effects.

Table 4

Comparison of Chemical Shifts in Solution and Solid State.

Assignments refer to Figure 13.

Solution State	Solid State	Assignment
9.2	6.9	25
9.3	10.1	32
9.4		33
9.5		28
19.3	18.4	24, 31
23.7	21.3	10
34.4	32.3	23, 30
99.2	101.1	5
100.1	119.6	15
117.2		21
119.0		13
119.8		7
122.1	124.4	6
122.2		26
122.3		14
122.6		18
123.4		2, 8
124.0		12
127.2	126.6	20
127.5		27
127.6		4
128.4		16
130.6	134.5	9
131.4		11
140.5	139.5	3
142.0		17
170.6	172.7	19
171.5		1
174.1	179.7	22, 29

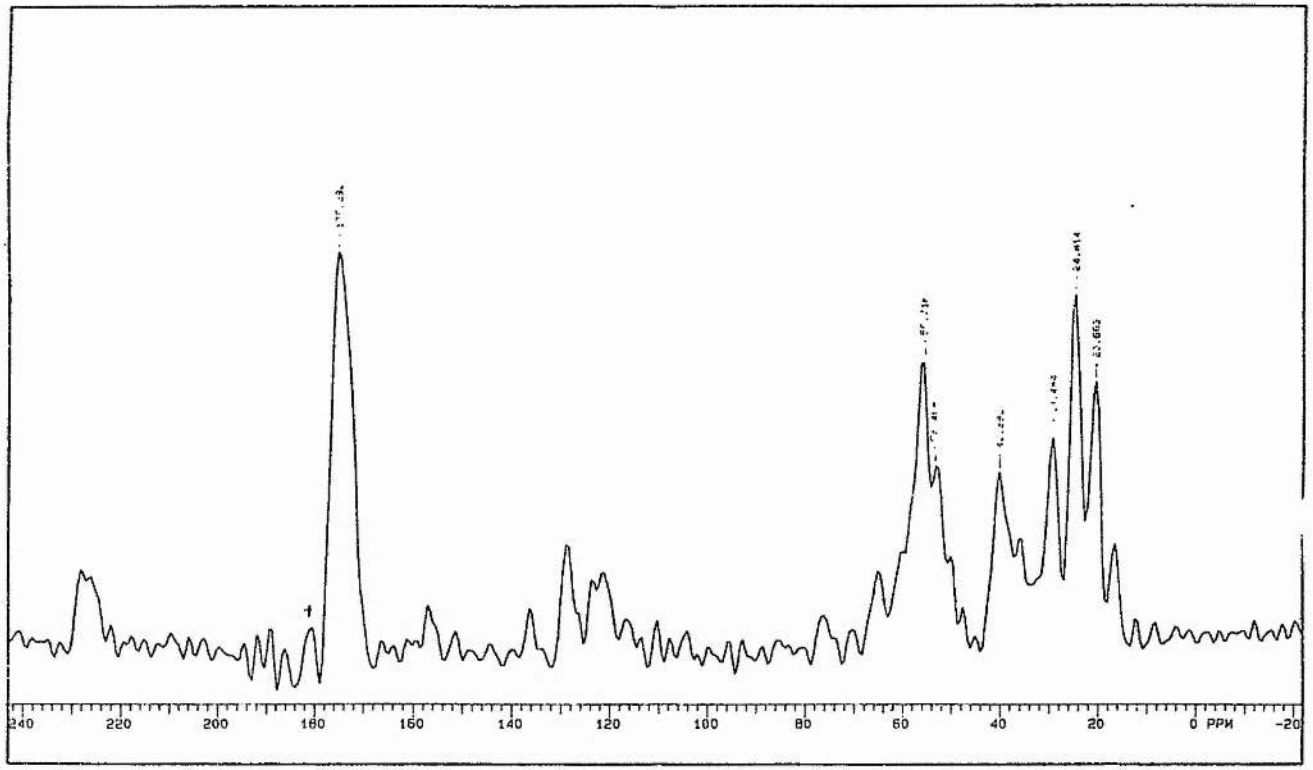


Figure 15 ^{13}C solid state NMR spectrum of bovine serum albumin using CPMAS mode with enhanced resolution.

^{13}C Solid State NMR of Bilirubin bound to the High Affinity Site of Bovine Serum Albumin

In an attempt to study any changes in ^{13}C chemical shifts due to the interaction of bilirubin at the high affinity site of BSA a CPMAS spectrum (with enhanced resolution) was recorded of BSA (Figure 15 and Table 5).

Table 5

All ^{13}C assignments are made relative to external TMS.

^{13}C Solid State NMR	
Albumin only	Albumin-Bilirubin (mole ratio 1:0.38)
16.00	17.32
20.66	21.47
24.81	25.63
29.48	29.54
36.00	37.44
40.38	40.67
53.45	54.00
55.72	56.87
118.00	119.00
121.00	123.12
124.00	
128.00	129.90
175.29	176.10
181.50	181.50

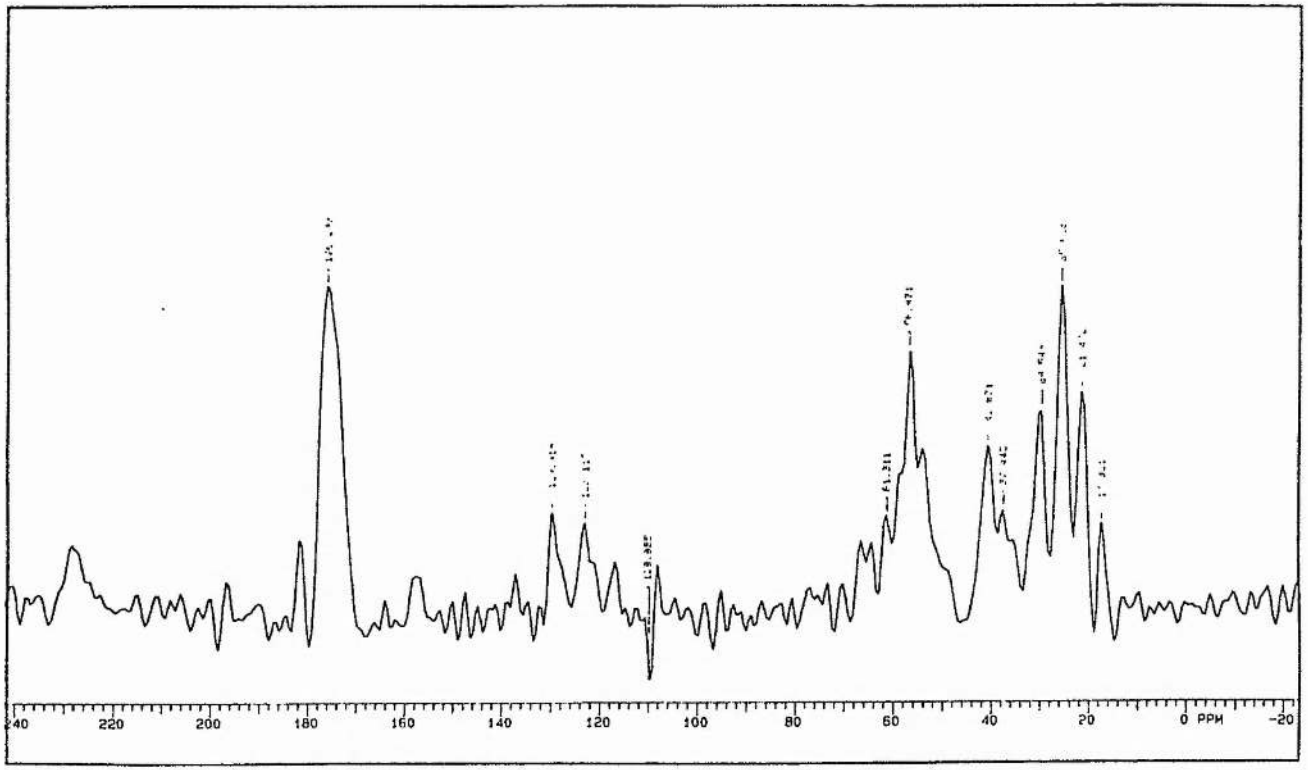


Figure 16 ^{13}C solid state NMR spectrum of 0.38:1 mole ratio bilirubin to bovine serum albumin.

An enhanced resolution CPMAS mode ^{13}C NMR spectrum was recorded of a sample containing a 0.38 mole ratio of bilirubin to BSA (Figure 16 and Table 5). Unfortunately to ensure the bilirubin is binding to the high affinity site this low mole ratio was used, this resulted in the ^{13}C solid state NMR spectrum being dominated by the resonances arising from BSA. However, comparing the chemical shifts of unbound and bound albumin shows definite changes of certain chemical shifts of saturated carbons. The resolution of the spectrum of bound albumin (see Figures 15 and 16) is less well defined and linewidths are in some cases broader indicating a decrease in crystallinity and thus binding. This preliminary ^{13}C solid state study of the binding of bilirubin to BSA indicates that in order to study the interaction at the high affinity site a labelled species of bilirubin is required to study direct changes in the bilirubin structure due to binding. The technique of ^{13}C solid state NMR using deuterated bilirubin in a CPMAS mode would give better resolved ^{13}C NMR spectra due to enhanced cross polarisation.

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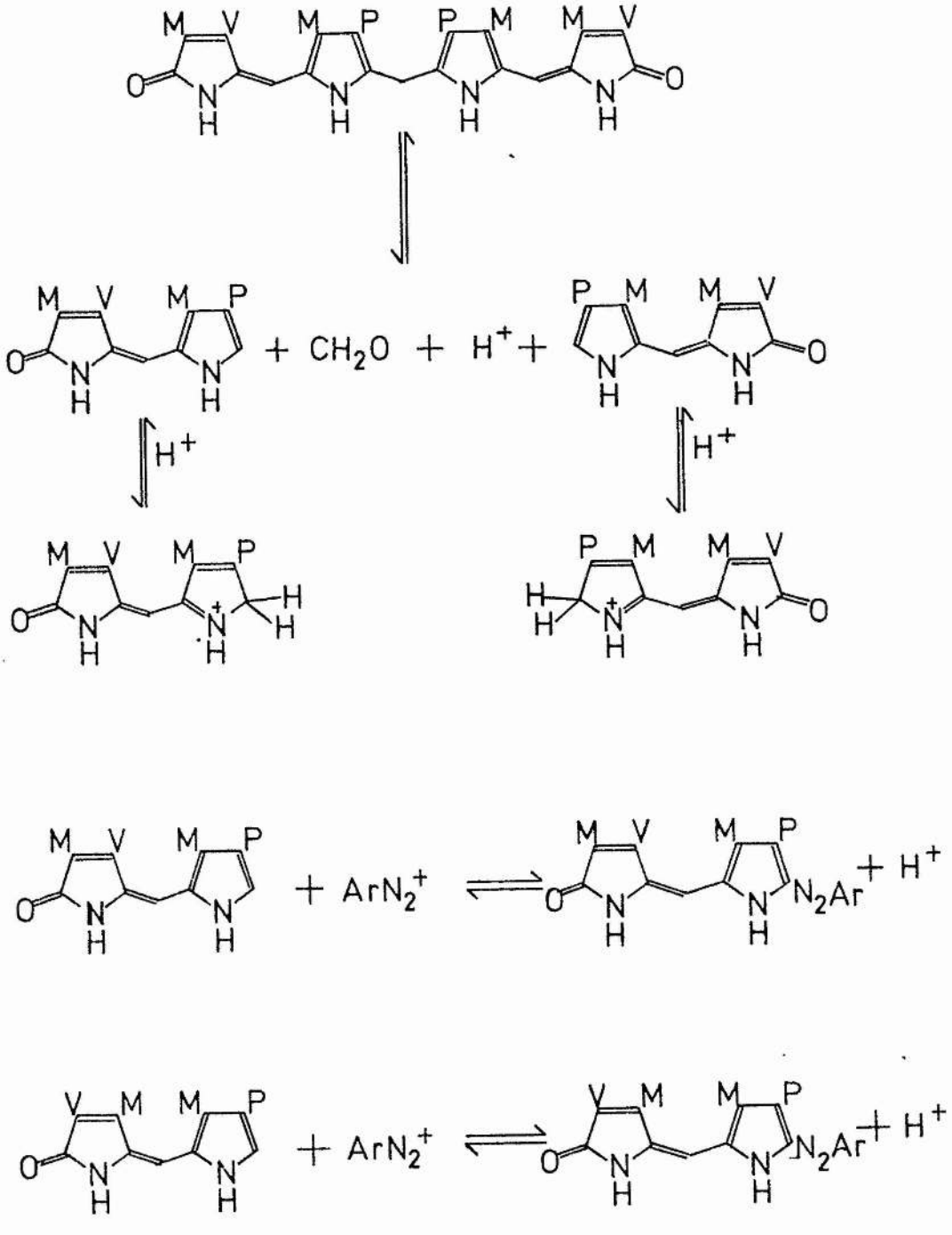
Chapter 7

PROBLEMS ASSOCIATED WITH THE DIAZO METHOD OF BILIRUBIN ASSAY



Scheme 1

7.1 INTRODUCTION

As discussed in Chapter 5 the most commonly used techniques for bilirubin assay are those based on van den Bergh's diazo-coupling method, *viz* the reaction between bilirubin and an excess of arenediazonium ion, p-diazobenzene sulphanilic acid. There have been many modifications[1-7] to the original test, e.g. various arenediazonium salts, pH changes, and "accelerators" (discussed in the preceeding Chapter). Of all of the modifications carried out one of the major factors influencing the rate of the diazo coupling reaction with bilirubin is pH. Landis and Pardue[8,9] have studied the kinetics of the van den Bergh test and it has been generally agreed that the reaction proceeds in two first order steps in the presence of an excess of p-diazobenzene sulphanilic acid (see Scheme 1). The reaction is first order in the reaction product, and is independent of the initial bilirubin concentration. Previous work[10] carried out in this department has shown that in the reaction of dipyrrylmethane with arenediazonium ion, a proton very rapidly cleaves the methylene bridge, and the methylene group forms formaldehyde. With bilirubin, the analogous reaction releases two oxydipyrromethene fragments, which then undergo a slower reaction with arenediazonium ions. Landis and Pardue[8] have studied the kinetics of the reaction in different pH solutions (pH 4-12) of bilirubin while keeping the arenediazonium ion in a strongly acidic medium. They found that the least protonated bilirubin species was the most reactive and that the most highly protonated species the least reactive. However, this appears to be due to the solubility profile of bilirubin at these different pH. They report problems of precipitation and data below pH 5 are only estimates for conjugated bilirubin. Therefore, in an attempt to investigate the effect of pH on the diazo coupling reaction of differ-

ent arenediazonium ions, a simple pyrrole model, 2,5-dimethylpyrrole, was used instead of bilirubin. From this present study using p-diazobenzene sulphanilic acid tetrafluoroborate salt it was noted that at higher pH a solution of this salt reacted rapidly, even in the absence of pyrrole, to produce a coloured product. Using uv-visible spectroscopy and ^{15}N NMR this apparent self-coupling reaction was investigated and its potential interference with bilirubin diazo coupling assessed. Using ^{15}N NMR a study was carried out to investigate the stereochemistry of the double bond of the azodipyrrole formed in the azo coupling reaction (see Scheme 1).

In recent years the demand for a rapid and convenient spot test for bilirubin assay has lead the Eastman-Kodak Company to develop a diazo based dry film for total bilirubin assay.[11-13] The diazonium salt used is 4-N-carboxymethylsulphonyl benzediazonium hexafluorophosphate (see Figure 1). This is required as a solubilised form of

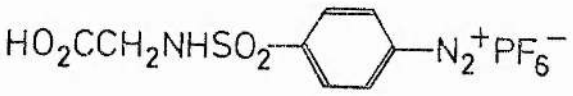


Figure 1 4-N-Carboxymethylsulphonyl benzediazonium hexafluorophosphate

diazonium ion which can be plated onto a multilayer film, and can couple with bilirubin to produce an azobilirubin (see Scheme 1) on the surface which can be quantitatively detected by visible spectroscopy. In this present work a preliminary study was carried out to investigate an alternative to the diazonium hexafluorophosphate salt and thus avoiding the use of unstable diazonium salts.

7.2 EXPERIMENTAL

7.2.1 KINETIC STUDIES INVESTIGATING THE EFFECT OF pH ON THE RATE OF REACTION OF 2,5-DIMETHYLPYRROLE WITH ARENEDIAZONIUM IONS

Materials

2,5-Dimethylpyrrole was purchased from Aldrich and redistilled and kept under nitrogen prior to use. p-Methoxyaniline, p-nitroaniline and sulphanilic acid were purchased from BDH and recrystallised or redistilled as appropriate before use. The benzenediazonium tetrafluoroborate salts of sulphanilic acid, p-methoxyaniline, and p-nitroaniline were prepared by the method of Starky.[14] For comparison of the kinetic reaction products, analytically pure samples of mono-azo-2,5-dimethylpyrroles were prepared by the use of dicyclohexyl-18-crown-6-ether as a phase transfer catalyst.[15]

Solutions of both 2,5-dimethylpyrrole and arenediazonium tetrafluoroborate were prepared in a solvent system of 75% aqueous buffer and 25% redistilled acetonitrile. This solvent system was prepared at various pH's by addition of acetonitrile to various pH buffers. The pH was then remeasured. Solutions of 2,5-dimethylpyrrole were 5×10^{-4} mol dm^{-3}. Both diazonium and pyrrole solutions were protected from light at all times, and kept cool prior to use.

Instrumentation

A Hi-Tech SF-3L stopped-flow spectrophotometer and SF-40C photomultiplier, Data-Lab DL 901 transient recorder, and a DT V12-14 Farnell oscilloscope were used for the kinetic studies of the reaction of 2,5-dimethylpyrrole with diazonium tetrafluoroborate salts of $p\text{-}NO_2C_6H_4N_2^+$ and $p\text{-}SO_2OHC_6H_4N_2^+$. An SP 8-100 uv-visible spectrophotometer was employed to study the reaction of 2,5-dimethylpyrrole and $p\text{-}MeOC_6H_4N_2^+$. Data acquisition and processing was carried out by an Apple II plus microcomputer using a Hi-Tech system software kinetics package. Rate constants were calculated by a computer program using the Kezdy[16]-Swinbourne[17] method. The reactions were all first order in the appearance of product; mono-azopyrrole.

The reactions were monitored at 425nm, and at 25°C. In the case of the stopped-flow spectrophotometer a solution of pyrrole was placed in one arm and the diazonium ion in the other, the absorption at 425nm being measured upon mixing. For each observed rate constant at least six runs were carried out where they all gave consistent results, and the observed rate constants are an average of these runs.

7.2.2 BENZENEDIAZONIUM ION REACTIVITY

UV-Visible Spectroscopy Studies

For these optical studies an SP 8-100 uv-visible spectrophotometer was employed. The diazotised p-sulphanilic acid tetrafluoroborate was prepared by the method of Starky.[14] Solutions of diazonium salt were 0.02mol dm^{-3} and spectra were recorded at 25°C.

u^{15}N NMR Spectroscopy Studies of ^{15}N-Labelled p-$SO_2OHC_6H_4N_2^+BF_4^-$ in Various pH Buffers

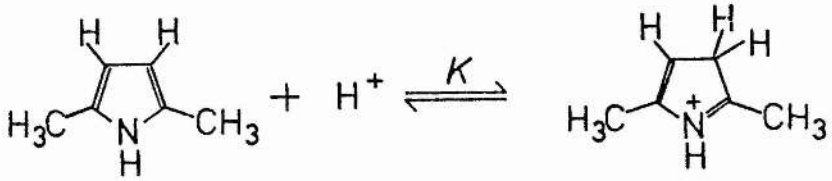
All ^{15}N NMR spectra were recorded in the FT mode at 25°C on a Bruker WH-360 spectrometer by the S.E.R.C. NMR Service at the University of Edinburgh. The spectra were recorded at 36.5MHz relative to CH_3NO_2 as the external reference, with spectral widths of 15151Hz, and a pulse delay of 0.35s between pulses of 4 seconds.

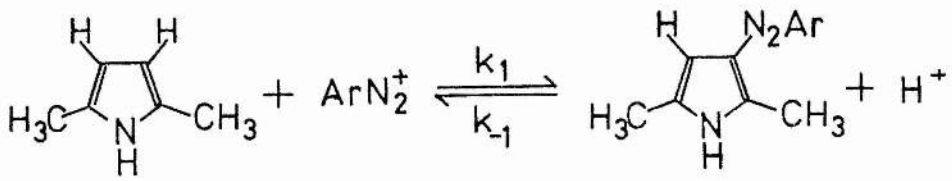
^{15}N-Labelled diazotised sulphanilic acid tetrafluoroborate salt was prepared by the method of Starky[14] using 98% sodium nitrite-^{15}N (Cambrian Gases). Solutions of ^{15}N-labelled diazonium salt were typically of a concentration of 0.054mol dm^{-3} in phosphate buffers (10%D_2O). ^{15}N-Labelled azobilirubin was prepared by the method of Kuenzle[18] using 90% $Na^{15}NO_2$. The phenylazobilirubin spectrum was recorded in chloroform-d at a concentration of 0.054mol dm^{-3}.

7.2.3 THE DEVELOPMENT OF A NEW DIAZO REAGENT FOR USE IN MULTILAYER FILM ASSAY OF BILIRUBIN

Materials

All the heterocyclic diazo compounds used in this study were kindly donated by Professor The Lord Tedder.





K (pyrrole basicity) = 0.195 l mol^{-1}

Scheme 2

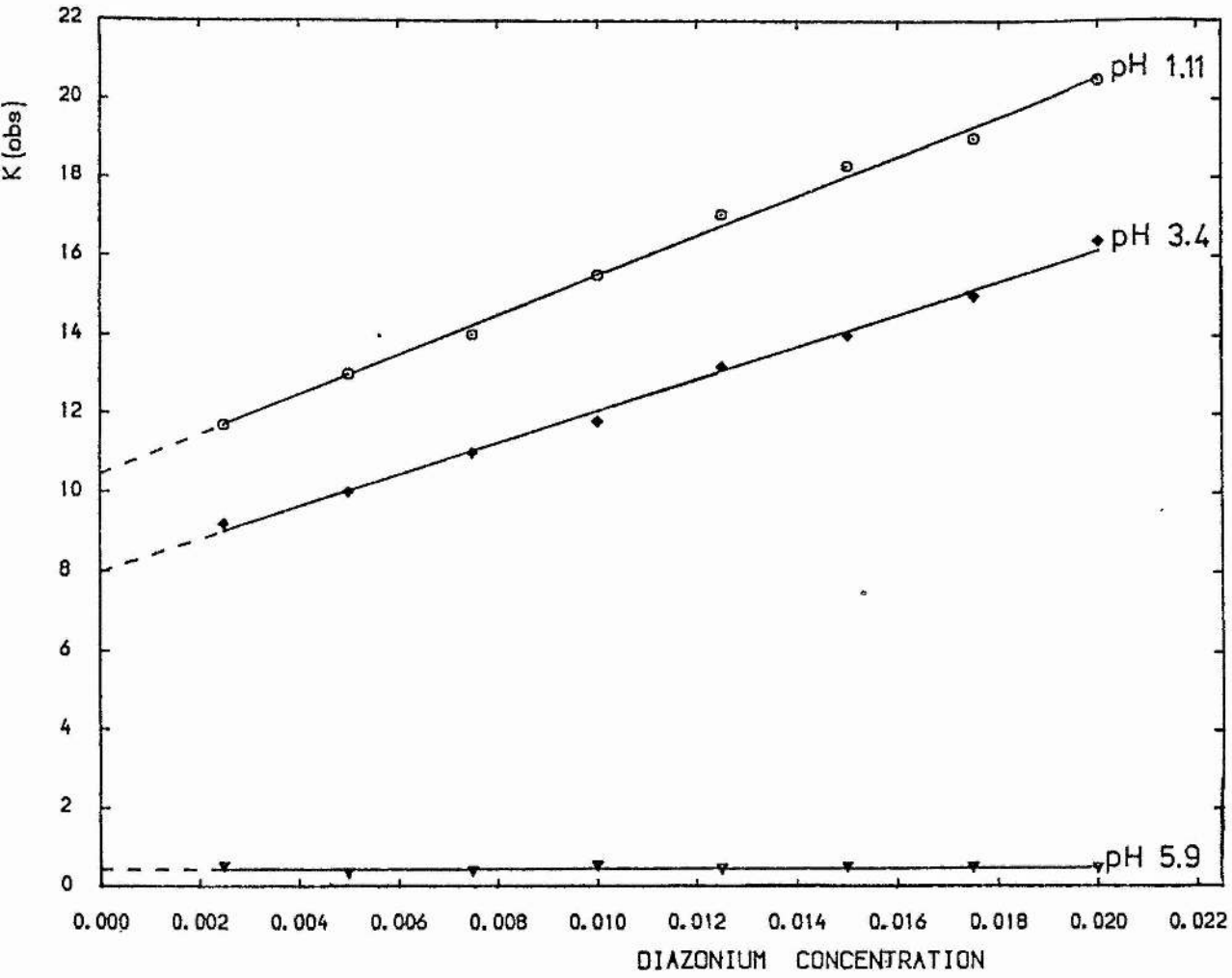
7.3 RESULTS AND DISCUSSION

7.3.1 KINETIC STUDIES INVESTIGATING THE EFFECT OF pH ON THE RATE OF THE REACTION OF 2,5-DIMETHYLPYRROLE WITH ARENEDIAZONIUM IONS

In this present study when the pH of both the van den Bergh[19,20] test and Evelyn-Malloy[21] method was monitored using human serum the resulting mixtures were found to be pH ~3. Carrying out a kinetic study of the reaction at pH $\leqslant$ 3 is complicated by the fact that bilirubin readily precipitates out of solution at that pH value. Previously, in an attempt to study the kinetics of azo coupling, simple pyrroles[22] and dypyrrylmethanes[10] were employed in the reaction with arenediazonium ions in strongly acidic conditions. The effect of pH on the rate of the reaction has not been studied. In this present work the effect of pH on the rate of the reaction between 2,5-dimethylpyrrole and three arenediazonium ions was investigated.

In the reaction of pyrroles with arendiazonium ions the pyrrole readily undergoes an electrophilic substitution and under acid or neutral conditions the product is an intensely coloured monoazopyrrole (see Scheme 2). In the case of 2,5-dimethylpyrrole the azo coupling occurs at the β position since the α is blocked. The reaction mechanism has previously been established[22] in acid conditions and was found to be first order in the appearance of product, and with a excess of arenediazonium ion, the rate was found to be independent of the initial pyrrole concentration. The actual azo coupling is an equilibrium process (see Scheme 2). The reaction is quite unusual in

Figure 2 Variation of k_{obs} with concentration of $p\text{-}NO_2C_6H_4N_2^+$ in the diazo coupling reaction with 2,5-dimethylpyrrole at 25°C and various pH values.



that in an already highly acidic environment one of the products of the reaction is the hydrogen ion (H^+). It has been established by previous workers[22] that the experimentally observed rate constant, k_{obs}, is given by :-

$$k_{obs} = \{k_1[ArN_2^+]/(K[H^+] +1)\} + k_{-1}[H^+]$$

If k_{obs} is plotted against $[ArN_2^+]$, i.e. the diazonium ion concentration, the intercept of the y-axis is equal to $(k_{-1}[H^+])$. The term $[H^+]$ was taken to be the molarity of the H^+ ions present at each pH. The basicity, K, of 2,5-dimethylpyrrole is 0.1951^{-1} mol^{-1}, obtained from Chiang and Whipple.[23] Initially the kinetics of the 2,5-dimethylpyrrole with p-nitrobenzene diazonium tetrafluoroborate at various pH values was studied and k_{obs} plotted against $[ArN_2^+]$ and the slope of the line, k_1, and k_{-1} were calculated (see Figure 2). Under similar conditions the reaction of 2,5-dimethylpyrrole and p-MeO-$C_6H_4N_2^+$ and p-$SO_2OHC_6H_4N_2^+$ were then investigated and k_1 and k_{-1} calculated for both. The values of k_1 and k_{-1} calculated are shown in Table 1.

Table 1

Data for the reactions of 2.5-dimethylpyrrole (5.4×10^{-4} mol dm^{-3}) with p-$X_6H_4N_2^+$ ions (0.02mol dm^{-3}) at various pH values at 25°C. All values of k_1 and k_{-1} are quoted as 1 mol^{-1} s^{-1}.

pH	X = NO_2		X = SO_2OH		X = MeO	
	k_1	k_{-1}	k_1	k_{-1}	k_1	k_{-1}
1.11	505	135	142	0	0.318	0
1.4			107	0		
3.4	412	1800			0.44	0
5.9	3.09	2100			0.7	0

In the case of the reaction of p-SO_2OH benzenediazonium tetrafluoroborate and 2,5-dimethylpyrrole at pH $\geq$ 3 the diazonium salt solution darkened rapidly and would not react to any significant degree with the pyrrole. Therefore, results could not be obtained from kinetic studies at higher pH values. This rapid colouration at pH $\geq$ 3 was investigated further in section 7.3.2. From the established reaction (Scheme 2) in strongly acidic conditions it is apparent that k_1 and k_{-1} should be independent of $[H^+]$, i.e. pH. Whereas from these results in the case of p-$NO_2C_6H_4N_2^+$ as pH increased there was a decrease in k_1 and a vast increase in k_{-1}. This trend is also reflected in the decrease of k_1 in the reaction of pyrrole and p-$SO_2OHC_6H_4N_2^+$. Whereas with p-$MeOC_6H_4N_2^+$ k_1 increased as pH was increased (see Table 1). These results are difficult to explain in terms of reaction Scheme 2, they must in fact be due to covalent interactions at higher pH of both p-$NO_2C_6H_4N_2^+$ and p-$SO_2OHC_6H_4N_2^+$ with the anion of the buffer causing a rate decrease in the coupling reaction to pyrrole. With p-$MeOC_6H_4N_2^+$ instead there is an increase in k_1, this may be due to salt effects on the reaction and will have to be considered further.

It is apparent from this set of experiments that although in strongly acidic conditions pyrroles undergo straightforward coupling reactions with arenediazonium ions, at a more realistic van den Bergh test pH ($\sim$3) the diazonium ion itself is subjected to other effects, e.g. covalent and salt interactions that may interfere with the azo coupling to pyrrole, or indeed bilirubin. These complications involved with diazonium ion coupling are also valid for bilirubin diazo

assay which is carried out routinely today, and may be one of the factors causing inconsistent quantitative results in bilirubin assay.

7.3.2 BENZENEDIAZONIUM ION REACTIVITY

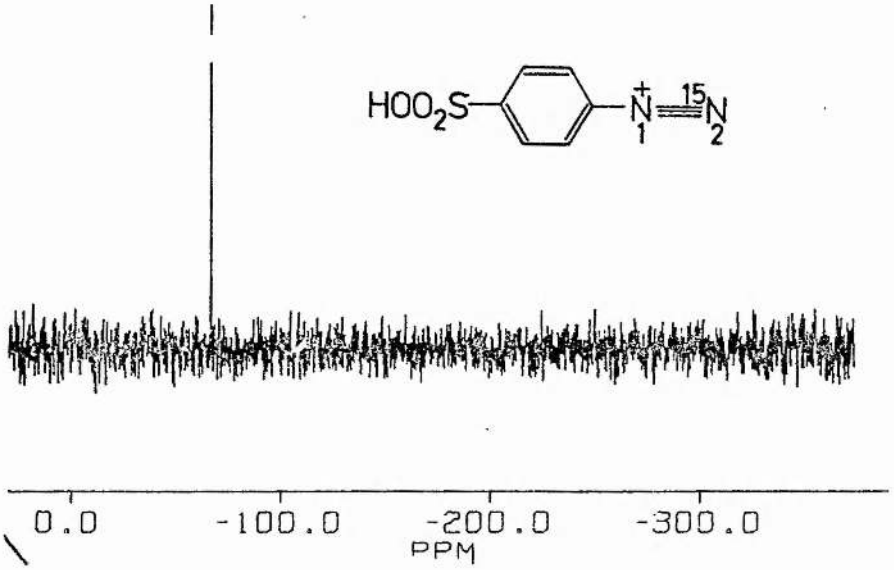
As noted in the reaction of 2,5-dimethylpyrrole and p-diazobenzene sulphanilic acid tetrafluoroborate salt at pH $\geq$ 3 the benzenediazonium tetrafluoroborate solution tends to darken and did not effectively react with the pyrrole. Modified van den Bergh methods use benzenediazonium ions at high pH.[4] Hence, this apparent benzenediazonium tetrafluoroborate self-coupling can seriously hinder any colourimetric procedure for bilirubin assay. To this end, initial uv-visible spectroscopy and ^{15}N NMR spectroscopy studies were carried out to investigate this rapid colouration at high pH ($\geq$ 3).

UV-Visible Spectroscopy Studies

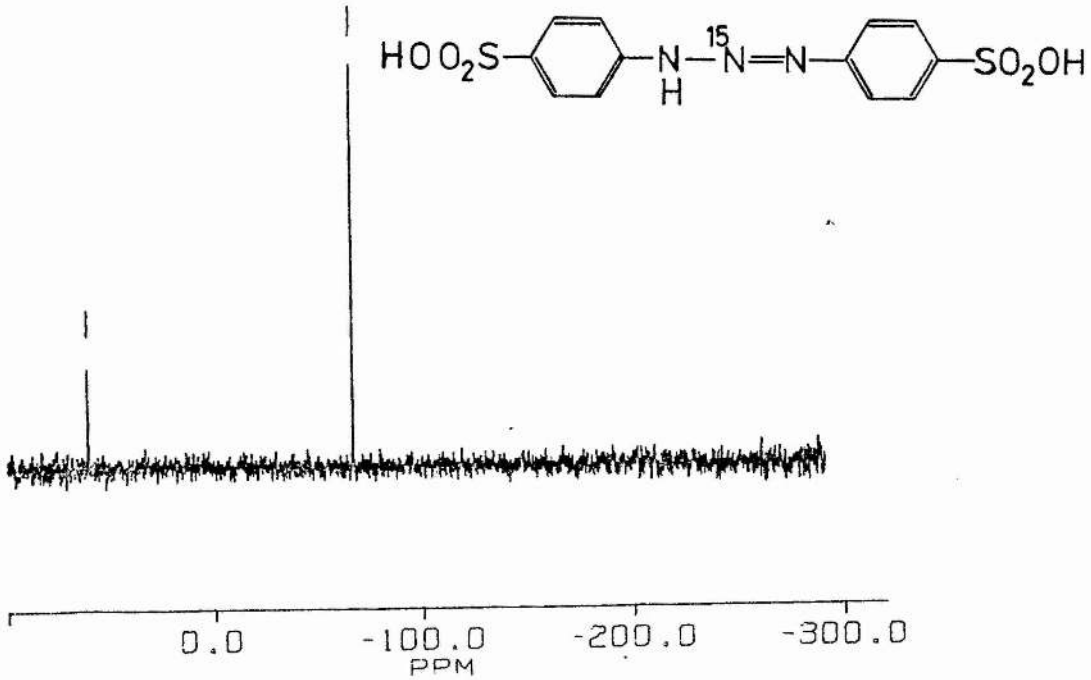
Solutions of p-diazobenzene sulphanilic acid tetrafluoroborate salt at various pH were left for a short period to react and their uv-visible absorptions are shown in Table 2.

Table 2

pH	uv absorption bands (nm)		
1.3	302	255	
4.0	355	310	
7.4	355	265	204



Figures 3a and 3b ^{15}N NMR spectrum of singly ^{15}N-labelled p-SO_2OH-$C_6H_4N_2^+$ in phosphate buffer pH 4.0 (3a) and pH 7.2 (3b).



At pH 1.3 two unsymmetrical sharp absorption bands (302 and 355nm) were observed. It has been reported that benzenediazonium tetrafluoroborate salts in acetonitrile absorb at 261 and 296nm[24], but for a para substituted species a single band is observed in the range 260-380nm. At pH 4.0 a broad band was observed at 355nm and a sharp symmetrical absorption at 310nm. When the pH was 7.4 three absorption bands became apparent, 355nm (broad), 265nm (sharp and symmetrical), and 204nm (sharp). It has been reported that triazenes[25] exhibit three absorption bands in the regions 230-240nm, 280-300nm, and 340-360nm. From these uv-visible spectral studies alone no definite conclusions can be made as to the species produced at different pH values.

^{15}N NMR Studies of ^{15}N-Labelled p-Diazonium-Benzene Sulphanilic Acid at Various pH Values

When the ^{15}N NMR spectrum of singly ^{15}N-labelled $SO_2OHC_6H_4N_2^+$ BF_4^- in buffer (pH 1.39) was recorded, a single ^{15}N resonance was observed at δ = -66.86ppm (see Figure 3a). This resonance is characteristic of the N(2) of the diazonium ion species. At pH 4.0 the ^{15}N NMR spectrum of the diazonium ion again only gave a ^{15}N resonance at δ = -66.86ppm. However, at pH 7.2 ^{15}N NMR spectrum gave two ^{15}N resonances at δ = +61.43 and δ = -66.36ppm. The resonance at δ = -66.36ppm arises from unreacted diazonium ion, but the new resonance at δ = +61.43ppm is characteristic of the central nitrogen atom of a triazene[26,27] (see Figure 3b). Therefore, when the pH of the solution containing the benzenediazonium tetrafluoroborate is raised

to pH 7.2 some triazene is detected by ^{15}N NMR spectroscopy. This triazene could be the result of the reaction of the diazonium ion with sulphanilic acid from either of two sources :-

(i) sulphanilic acid contaminating the sample from its preparation, which at pH 7.2 becomes deprotonated and therefore reactive, or

(ii) free sulphanilic acid amine could be the result of the reversal of the diazotisation reaction, i.e.

$$ArN_2^+ + H_2O \rightleftharpoons ArNH_2 + HNO_2 + H^+$$

However, on analysis, the spectrum obtained at pH 7.2 indicates the diazotisation is not reversible under these specific experimental conditions since the peak at δ = -66.86ppm did not completely disappear. Therefore, the amine required for triazene formation must in fact be a sulphanilic acid contaminant in the preparation of the diazonium tetrafluoroborate salt. In bilirubin assay the formation of the triazene as low as pH 7 could in colourimetric analysis lead to spurious results, unless the pH is kept low (< 3), or the absence of unreacted amine is certain.

These ^{15}N NMR studies also help clarify previous reports in the literature[28-34] of the formation of a spiro valence isomer of benzenediazonium ions which results in scrambling of the N(1) and N(2) atoms (see Figure 4).

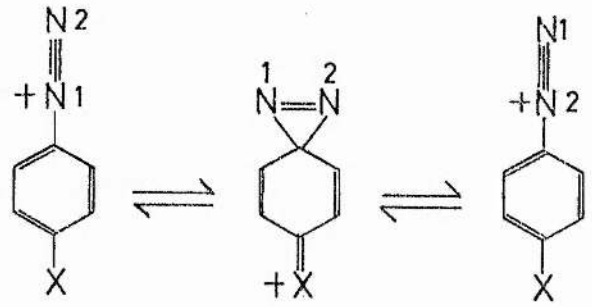


Figure 4 Mechanism of Nitrogen Scrambling.

Zollinger and his co-workers[28-30] have reported, from a series of experiments, evidence of nitrogen scrambling during dediazotisation. Previously, Insole and Lewis[31] from labelling experiments, and using mass spectrometry analysis of the nitrogen evolved after the reaction with azide, found evidence of nitrogen rearrangement accompanying decomposition of benzenediazonium ions. However, using nuclear spin-spin interaction between ^{1}H and ^{15}N after coupling with ethyl acetoacetate, Bose and Kugajevsky[32] could not confirm nitrogen scrambling. However, Lewis and Kotcher[33] using the same NMR techniques as Bose and Kugajevsky reaffirmed their earlier observations. In the present study the use of ^{15}N isotopic labelling of the benzenediazonium salt gave a sensitivity such that a 1% rearrangement could have been detected by the technique of ^{15}N NMR spectroscopy. Nevertheless, no scrambling was observed in the amine-diazonium coupling to form the triazene. These results are consistent with the recent work of Mitsuhashi *et al*[35] studying the mechanism of the reaction of 1,3-diaryltriazenes with tetracyanoethylene.

In the solid state[36] benzenediazonium ions are essentially linear and it is suggested that this valence isomer (see Figure 4) is a relatively high energy state from M.O. calculations.[37] Therefore, absence of nitrogen rearrangement is consistent with this data.

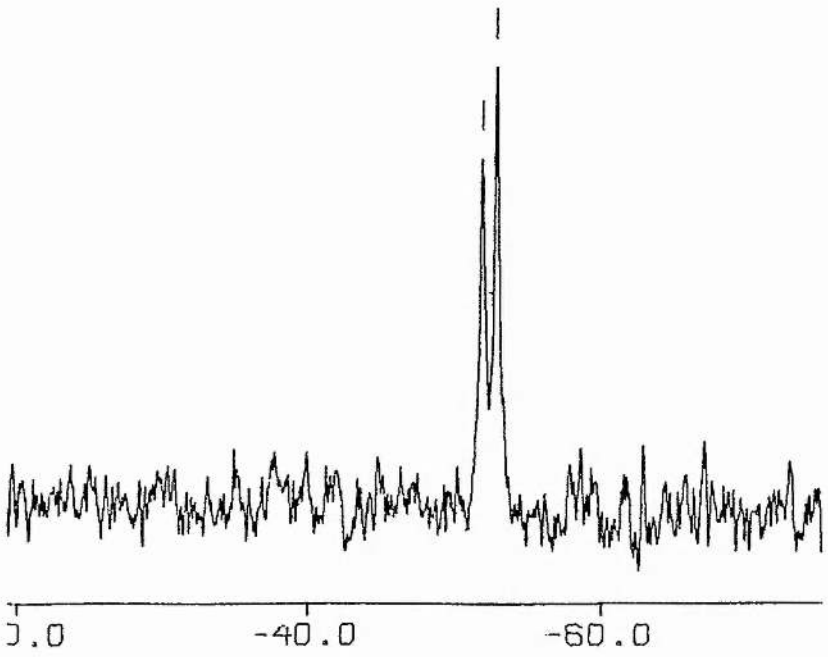


Figure 5 ^{15}N NMR spectrum of ^{15}N-labelled azobilirubin.

^{15}N NMR Study of the Stereochemistry of Azodipyrrole (Azobilirubin)

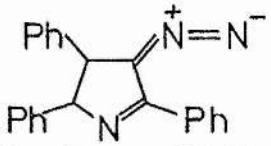
When bilirubin undergoes an azo coupling reaction with diazotised sulphanilic acid the product is azo-oxydipyrromethene; azobilirubin (see Scheme 1). The configuration of the azobilirubin across the double bond has as yet not been established. Due to steric effects it seems likely that the azo double bond adopts the anti configuration. To determine the actual configuration, ^{15}N-labelled azobilirubin was prepared by the reaction of ^{15}N-labelled phenyldiazonium chloride and bilirubin. When the ^{15}N-labelled azobilirubin ^{15}N NMR spectrum was recorded in chloroform-d two ^{15}N resonances were observed at δ = -51.98 and δ = -52.97ppm (see Figure 5). These correspond to both syn and anti conformational isomers of azobilirubin respectively. In chloroform the syn:anti ratio is 1:0.8, whereas in aqueous solution the in situ coupling of bilirubin and ^{15}N-labelled diazotised sulphanilic acid tetrafluoroborate only one ^{15}N resonance, δ = -73.15ppm corresponding to azobilirubin, is observed. Therefore, in a non-polar environment, i.e. chloroform both syn and anti conformational isomers are present to an approximately equal degree, whereas in an aqueous environment only the anti conformation is found. These differences must be due to the solvation effects of the water molecules favouring the anti conformation.

This work is still further evidence for no nitrogen scrambling of the benzenediazonium ion in the coupling reaction with bilirubin. This is in contrast to the views expressed by Hegarty[38] that benzenediazonium ions may undergo nitrogen rearrangement during reaction.

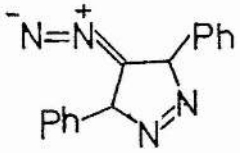
7.3.3 THE DEVELOPMENT OF A NEW DIAZO REAGENT FOR USE IN MULTILAYER FILM ASSAY OF BILIRUBIN

The Eastman-Kodak Research Laboratories have recently developed a diazo-based dry film[11,13] for the determination of total bilirubin in serum. When this film method for bilirubin was evaluated[12] it was found to have precision, reliability, and accuracy. It is thought to be a valid alternative to the wet chemistry methods of Jendrassik-Grof[3] or Evelyn-Malloy[21] assays for total bilirubin.

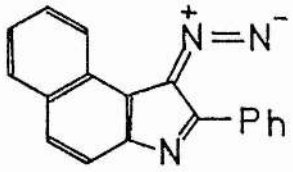
This multilayer film consists of a transparent support, "Estar" base, a gelatin Mordant Layer which contains pH 5 buffered gelatin, and a polymeric quaternary amine. The top layer consists of the metering, or spreading layer, of Triton X-100 surfactant and dyphylline (to dissociate the bilirubin from the serum albumin), and the diazonium salt; 4-(N-carbethoxymethylsulphamyl)benzenediazonium hexafluorophosphate (see p. 175). However, this new method still relies on diazonium salts which are unstable and as noted from previous sections can give rise to spurious results in coupling reactions. Instead of using diazonium salts, in this present work an initial study was carried out to investigate the use of heterocyclic diazo compounds, and evaluate their ability to azo couple with 2,5-dimethylpyrrole, 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane, and bilirubin.



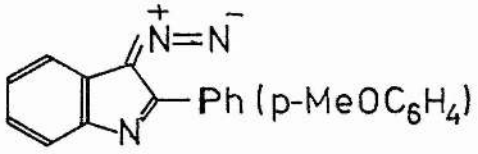
3-diazo-2,4,5-
triphenylpyrrole



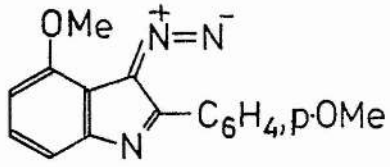
4-diazo-3,5-
diphenylpyrazole



3-diazo-2-phenyl-4,5-benzindole



3-diazo-2-phenyl(p-methoxyphenyl)-
indole



3-diazo-2-(p-methoxyphenyl)-
5-methoxyindole

Figure 7

It has been established[39,40] that the diazonium ion from nitrogen containing heterocycles can be readily converted by deprotonation into a stable heterocyclic diazo compound (see Figure 6). From previous studies[40] these compounds have been found to be retained on the surface of paper, and hence could potentially act as an indicator paper or "dipstick".

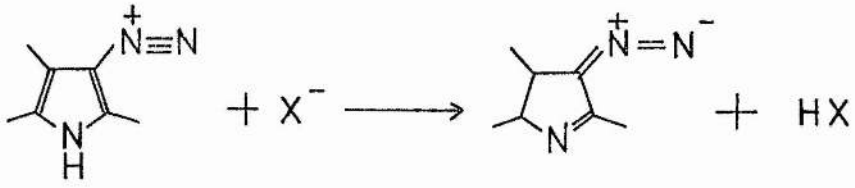


Figure 6 Diazotisation reaction of heterocyclic nitrogen compounds.

In an initial set of tests the reactivity of a number of heterocyclic diazo compounds (see Figure 7) with an acidified solution of 2,5-dimethylpyrrole was carried out. The most successful of the diazo compounds at azo coupling with 2,5-dimethylpyrrole was 3-diazo-2-phenylindole. A deep blood red solution was formed which had a λ_{max} at 512nm in the visible region. The other diazo compounds did not to any significant degree azo couple to the pyrrole.

In some cases of hyperbilirubinaemia levels of bilirubin in the human serum are found to be as high as 2.56×10^{-5} mol dm^{-3}. To determine whether 3-diazo-2-phenylindole can be successfully incorporated onto a test paper and give a positive test for bilirubin, strips of filter paper were dipped into an acidic solution of 3-diazo-2-phenylindole (4×10^{-3} mol dm^{-3}) and then dried. When these strips were dipped into an acidified solution of 3,3',4,4'-tetramethyl-5,5'-dicarboxy-2,2'-dipyrrylmethane (2.64×10^{-5} mol dm^{-3}) the paper

turned red. However, more success was found when the strips were dipped into an alkaline solution of bilirubin IXα-z,z (3.2×10^{-5}mol dm^{-3}), when the paper turned deep red. Therefore, at physiologically relevant concentrations of bilirubin this method can readily detect bilirubin without all of the inconvenience of the wet chemistry methods.[3,21] Why 3-diazo-2-phenylindole is more successful than other heterocyclic diazo compounds tested seems to be due to factors of solubility in aqueous media and the stabilising effect of electron donating substituents. Recently in the department[41] further studies have found 3-diazo-4-carbethoxypyrazole to be an excellent in azo coupling to N-methylpyrrole. Yet when in the present work 4-diazo-3,5-diphenylpyrazole was mixed with 2,5-dimethylpyrrole there was no significant azo coupling. This is further proof of the requirement for an electron withdrawing group to destabilise and activate the diazo substituent into an azo coupling reaction. Ideally, an excellent heterocyclic diazo compound for the azo coupling to bilirubin would be 3-diazo-4-cyanopyrazole, or 3-diazo-4-nitromethylpyrazole. These compounds could indeed be very successful, and readily incorporated into future multilayer dry films making bilirubin assay more accurate, precise, reliable, and most of all more convenient.

7.4 REFERENCES

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1. Carbon-13 Study of the Binding of Nitroprusside and Hexacyanoferrate(II) to Aquocobalamin, Vitamin B_{12a}.
   A.R. Butler, C. Glidewell, A.S. McIntosh, D. Reed, and I.H. Sadler; INORG. CHEM., 1986, 25, 970.
2. The Pharmacological Consequences of mixing Sodium Nitroprusside with Aquocobalamin or Thiosulphate.
   A.R. Butler, D.S. Hewick, P. Johnston, M. Mahoney, and A.S. McIntosh; BRIT. J. PHARMACOL. (Proceedings Supplement), 1986, 89, 508P.
3. Sodium Nitroprusside: Pharmacological Aspects of its Interaction with Aquocobalamin and Thiosulphate.
   D.S. Hewick, A.R. Butler, C. Glidewell, and A.S. McIntosh; J. PHARM. PHARMACOL., in the press.
4. Binding of Cyanoferrates to Cobaloximes and Cobalamins an EHMO Study.
   A.R. Butler, A.F. Cuthbertson, C. Glidewell, and A.S. McIntosh; INORG. CHEM. ACTA, in the press.
5. Benzenediazonium Ions: Structure, Complexation and Reactivity.
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6. Carbon-13 NMR and EPR Study of Reactions between Nitroprusside and Haemoglobins.
   A.R. Butler, C. Glidewell, I. Johnson, and A.S. McIntosh; submitted to INORG. CHEM.

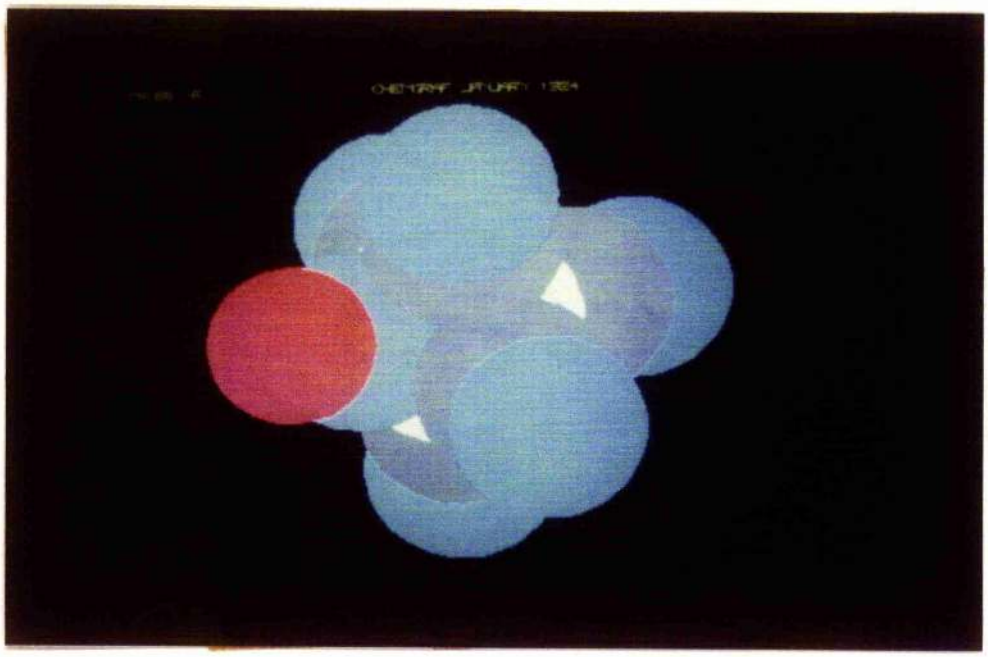
APPENDIX 1

Captions for Photographs

1. The Nitroprusside Anion $[Fe(II)(CN)_5NO]^{2-}$
2. Vitamin B_{12}
3. 1:1 Nitroprusside:Cobalamin Complex
4. 1:2 Nitroprusside:Cobalamin Complex
5. Space Filling Model of the 1:2 Nitroprusside:Cobalamin Complex
6. 1:1 Hexacyanoferrate(II):Cobalamin Complex
7. 1:2 Hexacyanoferrate(II):Cobalamin Complex
8. Aquomethylcobaloxime
9. Space Filling Model of Cobaloxime
10. 1:1 Nitroprusside:Cobaloxime Complex
11. 1:2 Nitroprusside:Cobaloxime Complex
12. 1:1 Hexacyanoferrate(II):Cobaloxime Complex
13. Space Filling Model of the 1:1 Hexacyanoferrate(II):Cobaloxime Complex
14. 1:2 Hexacyanoferrate(II):Cobaloxime Complex
15. Space Filling Model of the 1:2 Hexacyanoferrate(II):Cobaloxime Complex

16 and 17. Space Filling Model of the 1:2 Nitroprusside:Cobalamin Complex and L-Cysteine

18. Structure of Human Haemoglobin
19. Haem Moiety of the Human Haemoglobin α Subunit
20. Bilirubin IXα-z,z
21. The Intramolecular Hydrogen Bonded Structure of Bilirubin IXα-z,z
22. Ridge Tile Structure of Bilirubin IXα-z,z in the Solid State.



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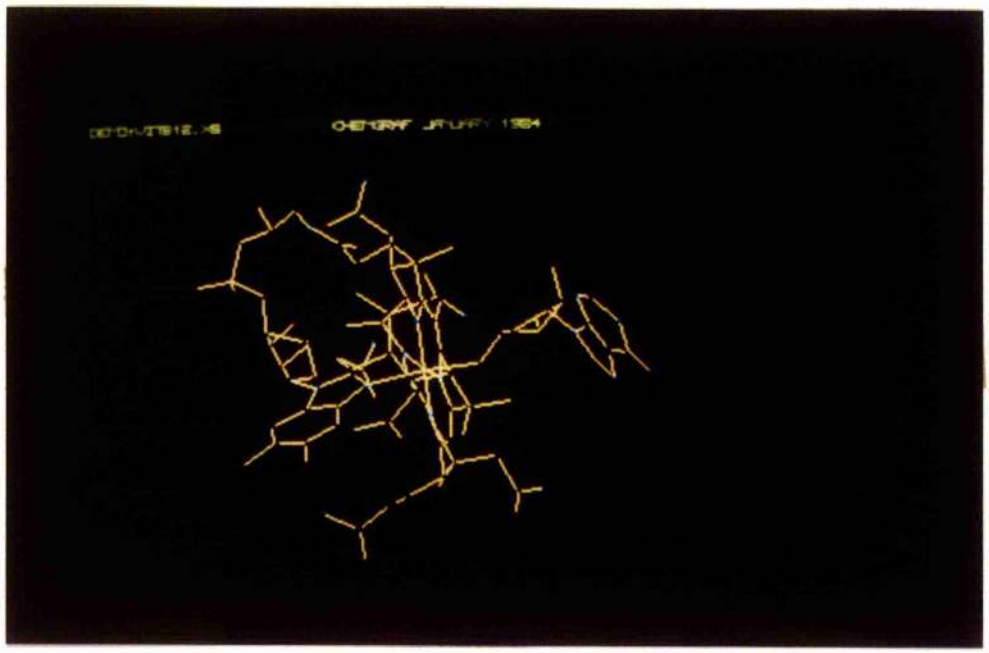

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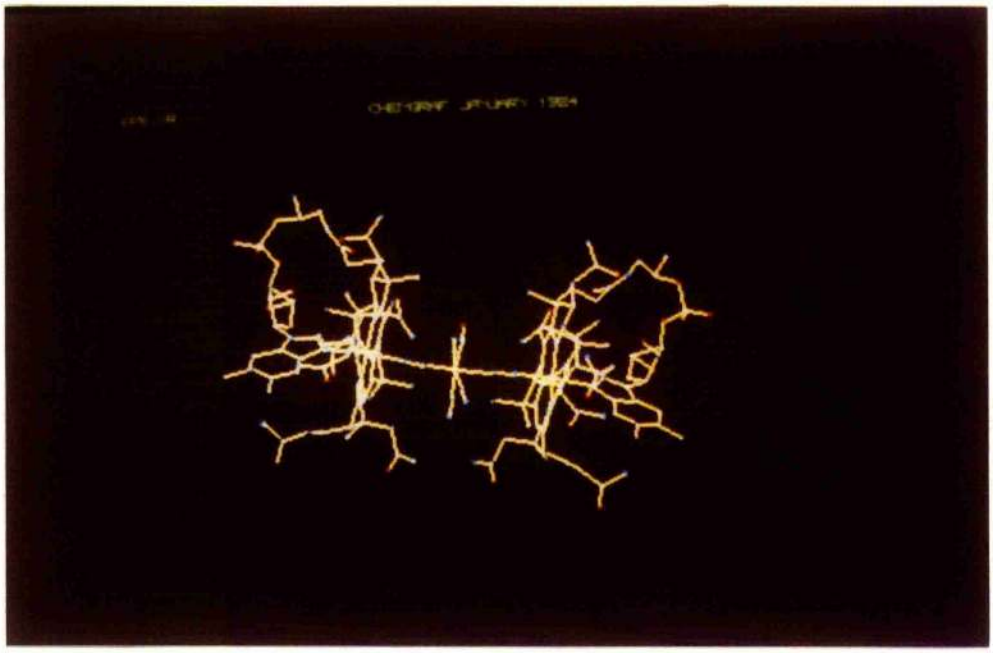

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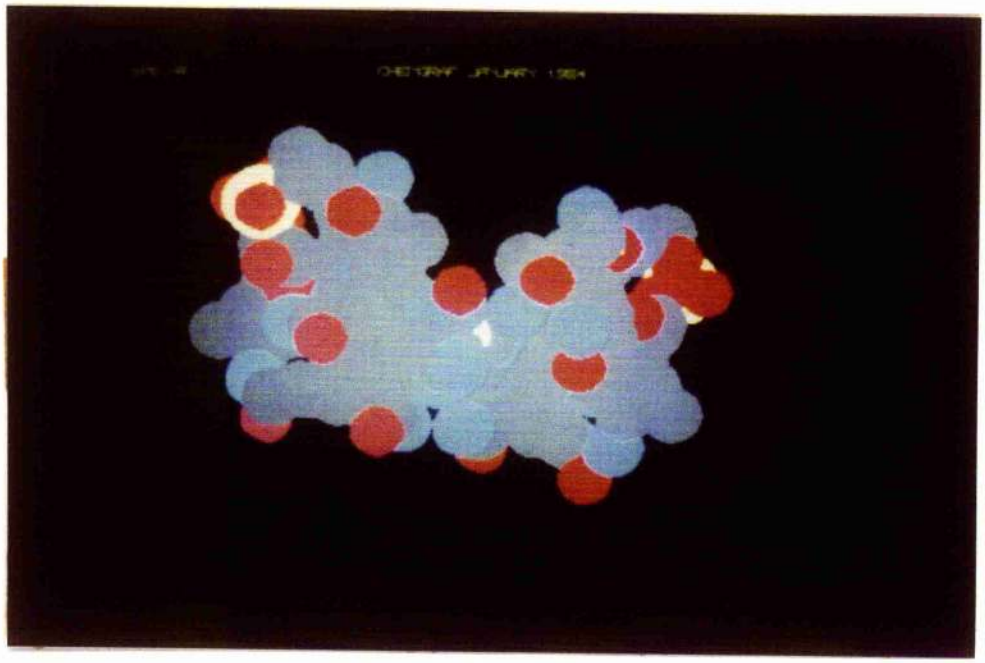

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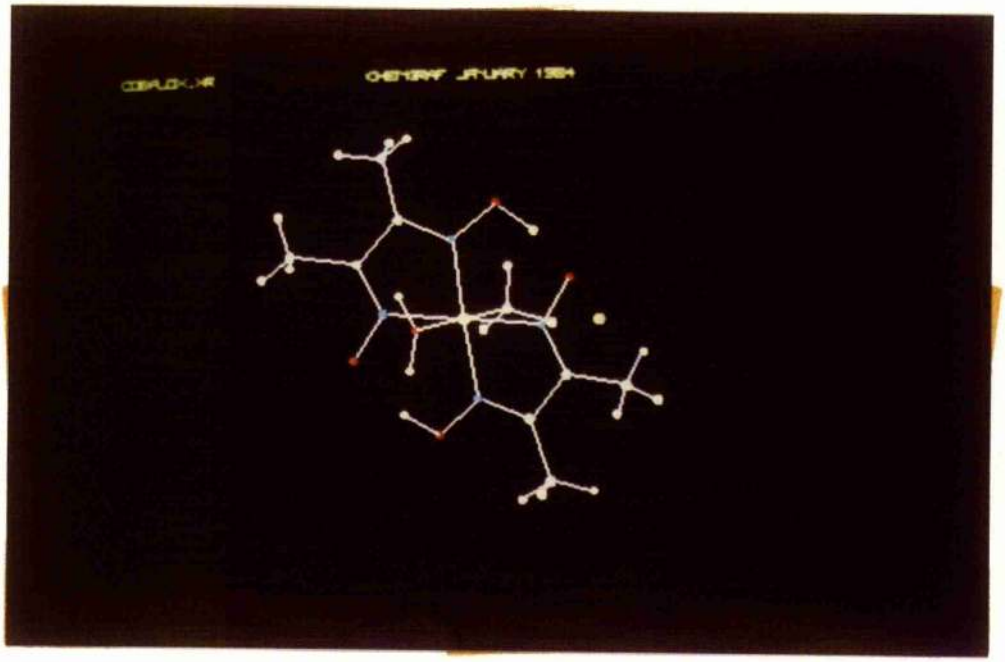

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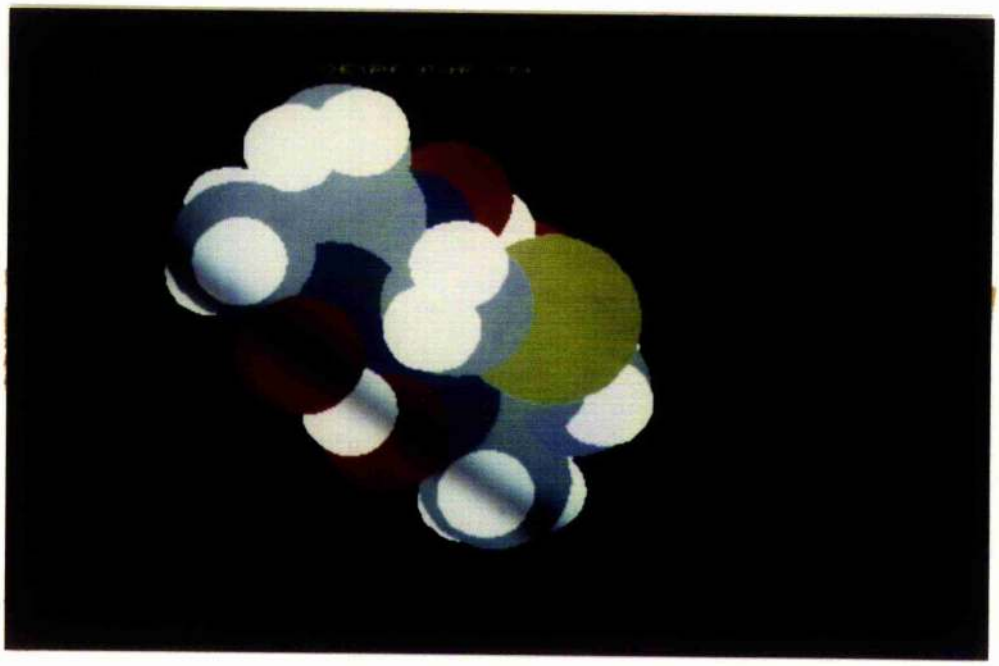

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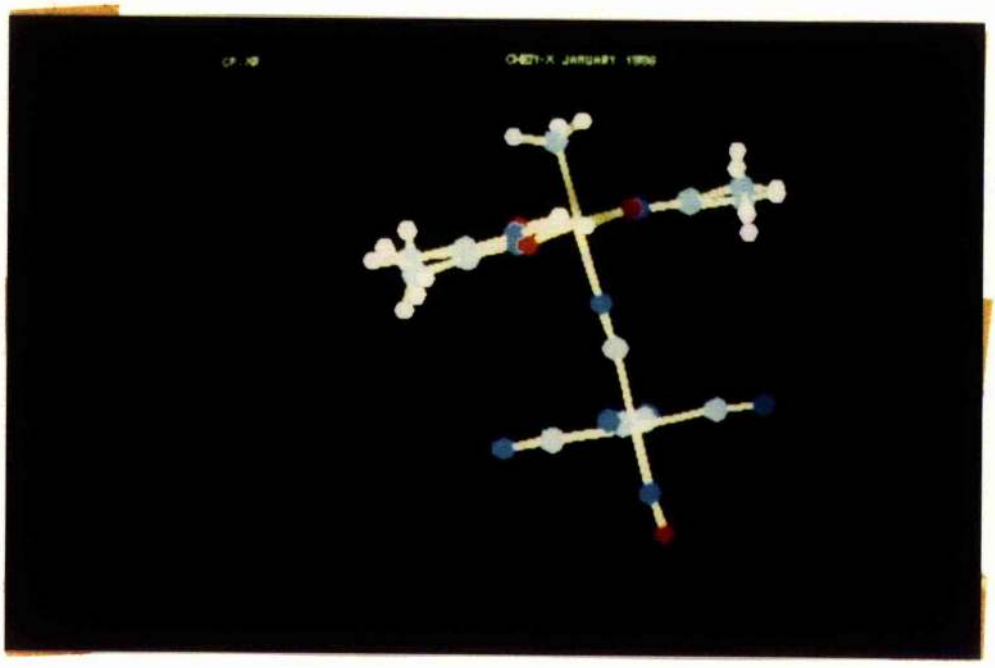

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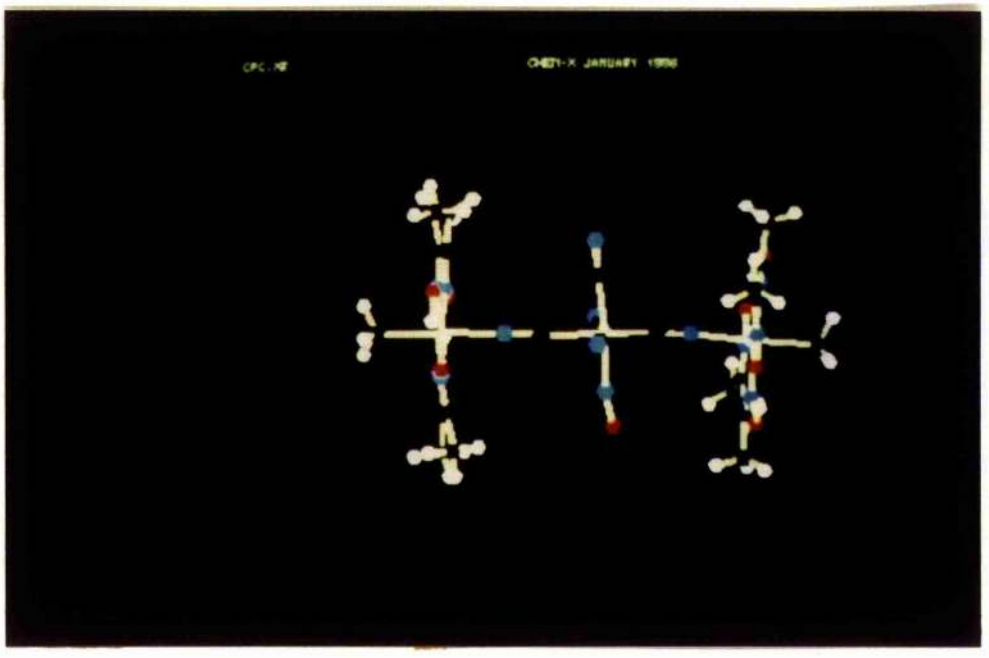

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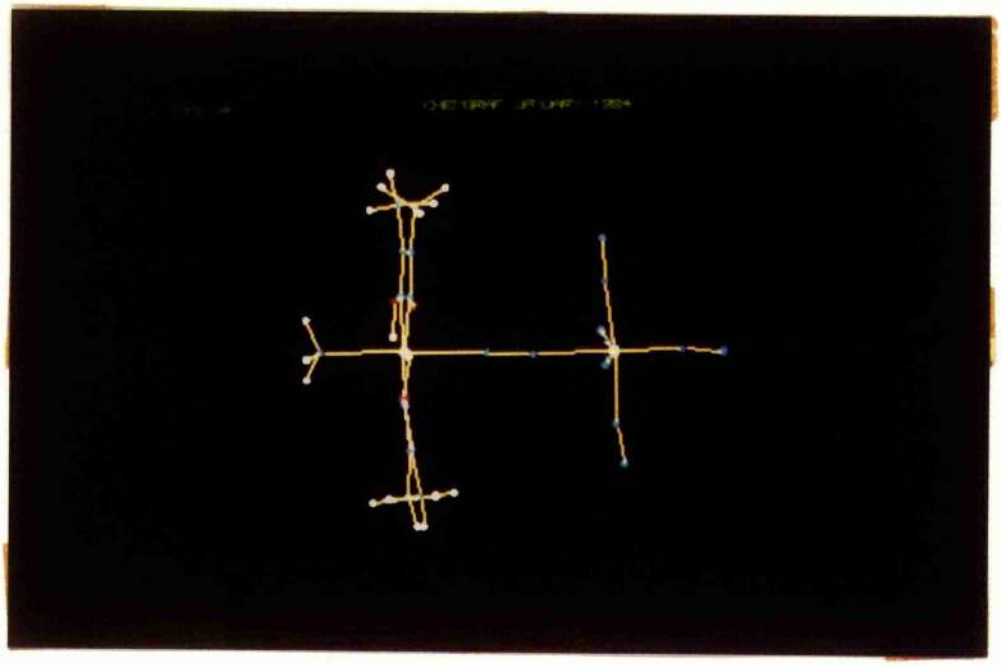

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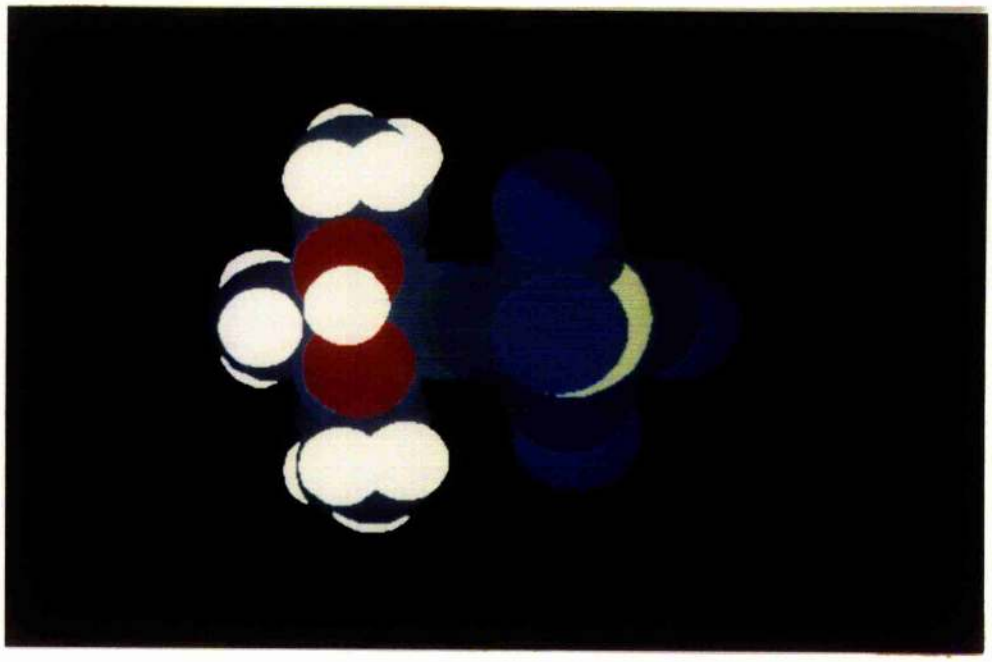

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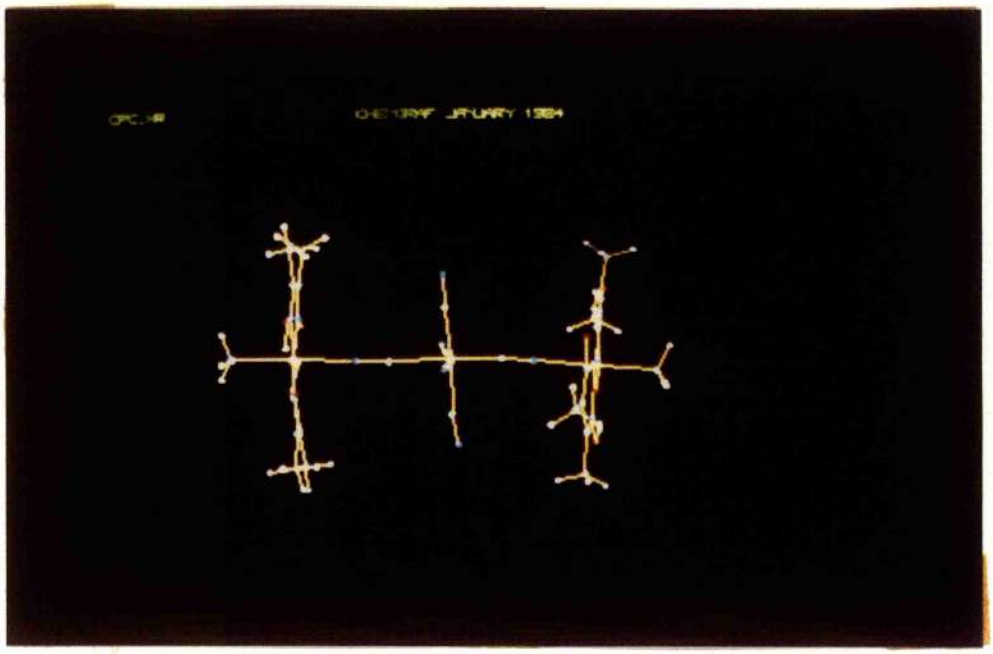

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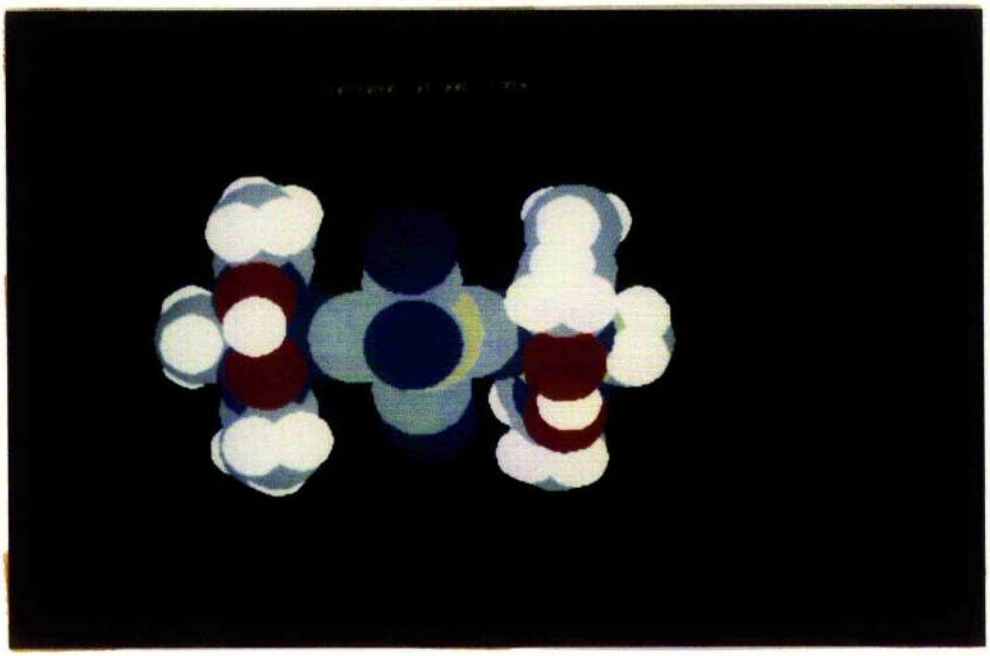

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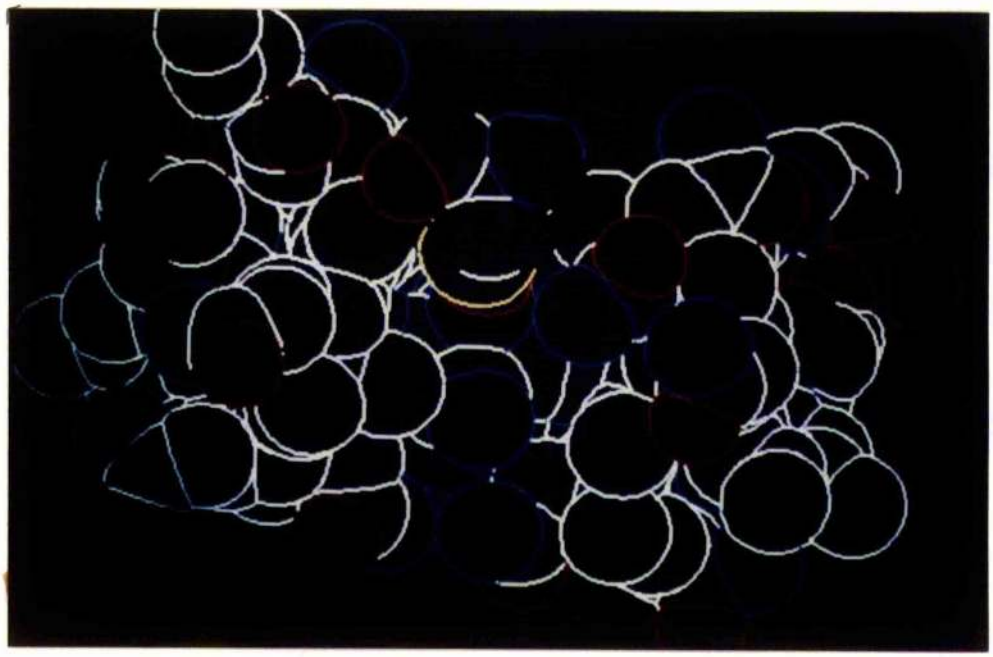

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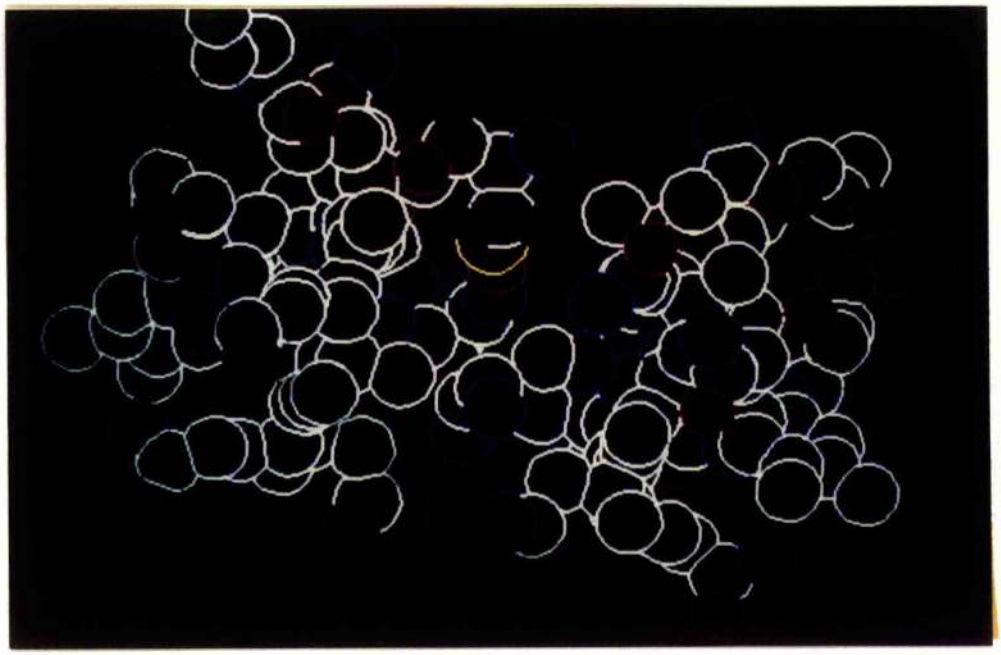

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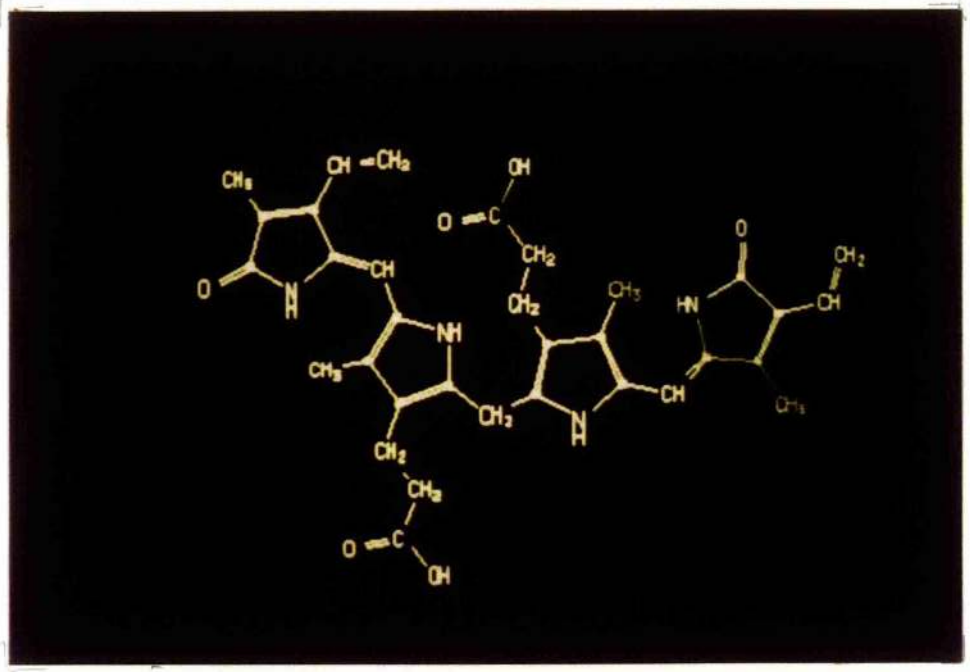
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OH
CH3
CH
N
H
HN
O
CH2
CH
CH3

APPENDIX 2

Protein Amino Acid Sequence

1. Human α Chain Haemoglobin

Composition

21 Ala A	1 Gln Q	18 Leu L	11 Ser S
3 Arg R	4 Glu E	11 Lys K	9 Thr T
4 Asn N	7 Gly G	2 Met M	1 Trp W
8 Asp D	10 His H	7 Phe F	3 Tyr Y
1 Cys C	0 Ile I	7 Pro P	13 Val V

Mol. Wt. unmod. chain = 15,126

Number of Residues = 141

```
                5         10        15        20        25        30
 1 V L S P A D K T N V K A A W G K V G A H A G E Y G A E A L E
31 R M F L S F P T T K T Y F P H F D L S H G S A Q V K G H G K
61 K V A D A L T N A V A H V D D M P N A L S A L S D L H A H K
91 L R V D P V N F K L L S H C L L V T L A A H L P A E F T P A
121 V H A S L D K F L A S V S T V L T S K Y R
```

2. Human β Chain Haemoglobin

Composition

15 Ala A	3 Gln Q	8 Leu L	5 Ser S
3 Arg R	8 Glu E	11 Lys K	7 Thr T
6 Asn N	13 Gly G	1 Met M	2 Trp W
7 Asp D	9 His H	8 Phe F	3 Tyr Y
2 Cys C	0 Ile I	7 Pro P	18 Val V

Mol. Wt. unmod. chain = 15,867

Number of Residues = 146

```
          5         10        15        20        25        30
 1 V H L T P E E K S A V T A L W G K V N V D E V G G E A L G R
31 L L V V Y P W T Q R F F E S F G D L S T P D A V M G N P K V
61 K A H G K K V L G A F S D G L A H L D N L K G T F A T L S E
91 L H C D K L H V D P E N F R L L G N V L V C V L A H H F G K
121 E F T P P V Q A A Y Q K V V A G V A N A L A H K Y H
```

3. Bovine α Chain Haemoglobin

Composition

20 Ala A	1 Gln Q	20 Leu L	13 Ser S
3 Arg R	5 Glu E	11 Lys K	8 Thr T
3 Asn N	9 Gly G	1 Met M	1 Trp W
8 Asp D	10 His H	7 Phe F	3 Tyr Y
0 Cys C	0 Ile I	6 Pro P	12 Val V

Mol. Wt. unmod. chain = 15,053

Number of Residues = 141

```
          5         10        15        20        25        30
 1 V L S A A D K G N V K A A W G K V G G H A A E Y G A E A L E
31 R M F L S F P T T K T Y F P(H.F)D L S H G S A Q V K G H G A
61 K V A A A L T K A V E(H.L.D.D)L P G A L S E L S D L H(A.H)K
91 L R V D P V N F K L L.S H S L L.V T L.A S H L P S D F.T P A
121 V H.A S L D K F L A N V(S.T.V)L.T S K Y R
```

```
                5         10        15        20        25        30
  1 M K W V T F I S L L F L F S S A Y S R G V F R R D A H K S E
 31 V A H R F K D L G E E N F K A L V L I A F A Q Y L Q Q C P F
 61 E D H V K L V N E V T E F A K T C V A D E S A E N C D K S L
 91 H T L F G D K L C T V A T L R E T Y G E M A D C C A K Q E P
121 E R N E C F L Q H K D D N P N L P R L V R P E V D V M C T A
151 F H D N E E T F L K K Y L Y E I A R R H P Y F Y A P E L L F
181 F A K R Y K A A F T E C C Q A A D K A A C L L P K L D E L R
211 D E G K A S S A K Q R L K C A S L Q K F G A R A F K A W A V
241 A R L S Q R F P K A E F A E V S K L V T D L T K V H T E C C
271 H G D L L E C A D D R A D L A K Y I C E N Q D S I S S K L K
301 E C C E K P L L E K S H C I A E B E N D E M P A D L P S L A
331 A D F V E S K D V C K N Y A E A K D V F L G M F L Y E Y A R
361 R H P D Y S V V L L L R L A K T Y E T T L E K C C A A A D P
391 H E C Y A K V F D E F K P L V E E P Q N L I K Q N C E L F K
421 Q L G E Y K F Q N A L L V R Y T K K V P Q V S T P T L V E V
451 S R N L G K V G S K C C K H P E A K R M P C A E D Y L S V V
481 L N Q L C V L H E K T P V S D R V T K C C T E S L V N R R P
511 C F S A L E V D E T Y V P K E F N A E T F T F H A D I C T L
541 S E K E R Q I K K Q T A L V E L V K H K P K A T K E Q L K A
571 V M D D F A A F E E K C C K A D D K E T C F A E E G K K L V
601 A A S Q A A L G L
```

6. Bovine Serum Albumin Precursor

Composition

47 Ala A	19 Gln Q	65 Leu L	32 Ser S
26 Arg R	59 Glu E	60 Lys K	35 Thr T
12 Asn N	17 Gly G	5 Met M	3 Trp W
39 Asp D	17 His H	30 Phe F	20 Tyr Y

35 Cys C 15 Ile I 28 Pro P 38 Val V

3 Asx B 1 Glx Z

Mol. Wt. unmod. chain = 69,161

Number of Residues = 606

```
          5         10        15        20        25        30
  1 N K W V T F I S L L L L F S S A Y S R G V F R R D T H K S E
 31 I A H R F K D L G E E H F K G L V L I A F S Q Y L Q Q C P F
 61 D E H V K L V N E L T E F A K T C V A D E S H A G C E K S L
 91 H T L F G D E L C K V A S L R E T Y G D M A D C C E K E Q P
121 E R N E C F L S H K D D S P D L P K L P K D P N T L C D E F
151 K A D E K K F W G K Y L Y E I A R R H P Y F Y A P E L L Y A
181 N K Y N G V F Q E C C Q A E D K G A C L L P K I E T N R E K
211 V L T S S A R Q R L R C A S I Q K F G E R A L K A W S V A R
241 L S Q K F P K A E F V E V T K L V T D L T K V H K E C C H G
271 D L L E C A D D R A D L A K Y I C B B Z B T I S S K L P E C
301 K D P C L L E K S H C I A E V E K D A I P E D L P P L T A D
331 F A E D K D V C K N Y Q E A K D A F L G S F L Y E Y S R R H
361 P E Y A V S V L L R L A K E Y E A T L E E C C A K D D P H A
391 C Y T S V F D K L D H L V D E P Q N L I K Q N C D Q F E K L
421 G E Y G F Q N A L I V R Y T R K V P Q V S T P T L V E V S R
451 S L G K V G T R C C T K P E S E R M P C T E D Y L S L I L N
481 R L C V L H E K T P V E S K V T K C C T E S L V N R R P C F
511 S A L T P D E T Y V P K A F D E K L F T F H A D I C T L P D
541 T E K Q I K K Q T A L V E L L K H K P K A T E E Q L K T V N
571 E N F V A F V D K C C A A D D K E A C F A V E G P K L V V S
601 T Q T A L A
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